The effects of quercetin on iron metabolism

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The effects of quercetin on iron metabolism

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Bismillah hir-Rahman nir-Rahim

Dedicated to my Mum and Dad for their endless love, support and supply of food!

And my “twin”/little sister Farzana for always making me smile.

Abstract

Polyphenols are known to be major inhibitors of dietary non-haem iron bioavailability, mainly through their action as iron chelators. In this present study Caco-2 cells were used to investigate the influence of quercetin, the most abundant flavonol in the diet, on non-haem iron bioavailability using ^{55}Fe, and the gene expression of intestinal iron transporters as measured via qPCR.

Chronic exposure to quercetin (24 hours) had no significant effect on iron uptake but iron efflux was significantly decreased. Consistent with this, qPCR analysis revealed a significant decrease in basolateral transport genes ferroportin (FPN) and hephaestin expression suggesting polyphenols may have direct gene regulatory effects. Exploring the cellular mechanisms underlying quercetin-induced FPN down-regulation, transfection of 5'FPN promoter constructs showed quercetin did not affect activity but did decrease FPN1A mRNA whilst increasing FPN1B expression; this suggests that although FPN1B is specific to intestinal epithelial cells, FPN1A remains the major isoform. FPN 3'UTR miRNA array analysis identified candidate hsa-miR-17-3p to be significantly activated by quercetin (1.5 fold) and qPCR validation confirmed up-regulation of 101 ± 25.1 -fold ($p<0.01$). This represents a novel mechanism of quercetin-induced miRNA-mediated regulation of FPN.

In HepG2 cells quercetin stimulated hepcidin expression and inhibited ferroportin gene expression; this may provide an additional means of regulating systemic iron levels. Quercetin was shown to be both pro- and anti- proliferative/apoptotic dependent on the concentration used which may have beneficial consequences for liver pathology of iron-overload diseases.

In contrast to findings in Caco-2 cells, in Thp1 macrophages quercetin caused a significant dose-dependent increase in FPN expression. Furthermore, quercetin induced both FPN1A and 1B promoter activities. This strongly implies that quercetin acts at the level of the FPN promoter to increase FPN expression - an effect specific to macrophages only. This demonstrates that quercetin has cell-specific effects and its actions on FPN are differentially regulated dependent on cell/tissue type.

The results show that quercetin can have multiple effects on iron homeostasis. Given its relatively long half-life in the circulation, repeated dietary intake of quercetin could lead to plasma accumulation *in vivo*. This may have important consequences for conditions that are low in iron such as anaemia; alternatively it has therapeutic potential for iron overload diseases such as haemochromatosis. By deducing the mechanisms of how dietary polyphenols interact with our intake of essential nutrients such as iron, intake can be optimised to harness the potential benefits polyphenols have to offer.

Acknowledgements

First and above all I thank God for making all things possible.

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Abbreviations

18S	18S ribosomal RNA
a.u.	Arbitrary unit
APS	Ammonium persulphate
BLAST	Basic local alignment search
BMP	Bone morphogenetic protein
Bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary
Cu	Copper
Dcytb	Duodenal cytochrome b
DFO	Deferoxamine
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DPM	Disintegration per minute
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
EPOR	Erythropoietin receptor
FAC	Ferric ammonium citrate
Fe	Iron
FPN	Ferroportin
GLUT	Glucose transporter
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Heph	Hephaestin
HH	Hereditary haemochromatosis
HIF	Hypoxia-inducible factor
HJV	Hemojuvelin
HRP	Horseradish peroxidase

IL	Interleukin
IRE	Iron responsive element
IREG1	Iron-regulated transporter 1
IRP	Iron regulatory protein
JAK	Janus kinase
JH	Juvenile haemochromatosis
kDa	Kilodalton
LIP	Labile iron pool
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
miRNA	Micro RNA
MRP2	Multidrug resistance-associated protein 2
Nramp1	Natural resistance-associated macrophage protein 1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
p-STAT	Phospho-STAT
PMA	phorbol myristate acetate
qPCR	quantitative polymerase chain reaction
RLU	Relative light unit
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SLC40A1	Solute carrier family 40,
STAT	Signal transducers and
STEAP	Six-transmembrane epithelial antigen of the prostate
TEMED	N,N,N′,N′-Tetramethylethylenediamine
Tf	Transferrin
TfR	Transferrin receptor
TRPML	Transient receptor potential cation channel, mucolipin subfamily
UTR	Untranslated region

1. Introduction

1.1. General iron introduction

Iron is one of the most important trace metals required for human life due to its fundamental roles in basic cell biology. It is involved in DNA synthesis and electron transport in mitochondria as well as being the main component of haemoglobin in red blood cells and myglobin in muscle. Its ability to exist in both the ferric (Fe^{3+}) and ferrous (Fe^{2+}) state enables it to take part in many redox reactions in the body. Through the Fenton- Haber-Weiss reaction free radicals can be generated, which are critical in defending the body against foreign pathogens.

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ Fenton reaction

$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$ Haber-Weiss reaction

However, excess tissue iron can cause oxidative stress, damaging cellular macromolecules including protein, DNA and mitochondria leading to cell death and tissue injury. There are no physiological means of excreting large amounts of iron thus iron homeostasis is primarily regulated at the dietary absorption stage. As both iron excess and deficiency can lead to cell damage, the balance of body iron levels must be tightly controlled.

1.2. Iron homeostasis

The human body contains approximately 3 - 5 g of iron (45 - 55 mg/kg of body weight). 60 – 70% of total body iron can be found in haemoglobin of circulating red

blood cells (Andrews 1999a). Iron-rich organs include muscle and liver, which has a dual role in iron metabolism of iron storage and hepcidin synthesis which is discussed later. A major supply of iron is from macrophages which are responsible for degrading haemoglobin from senescent erythrocytes and releasing iron back in to the circulation. This demonstrates an efficient cycle of iron re-utilisation, which ensures that only small amounts of iron are lost through minor bleeding and mucosal sloughing (Green et al. 1968). It is necessary to replace this loss and so although intestinal absorption might contribute to a small proportion of body iron, the 1-2 mg/day of dietary iron absorbed by duodenal enterocytes is essential for homeostatic balance (Figure 1.1).

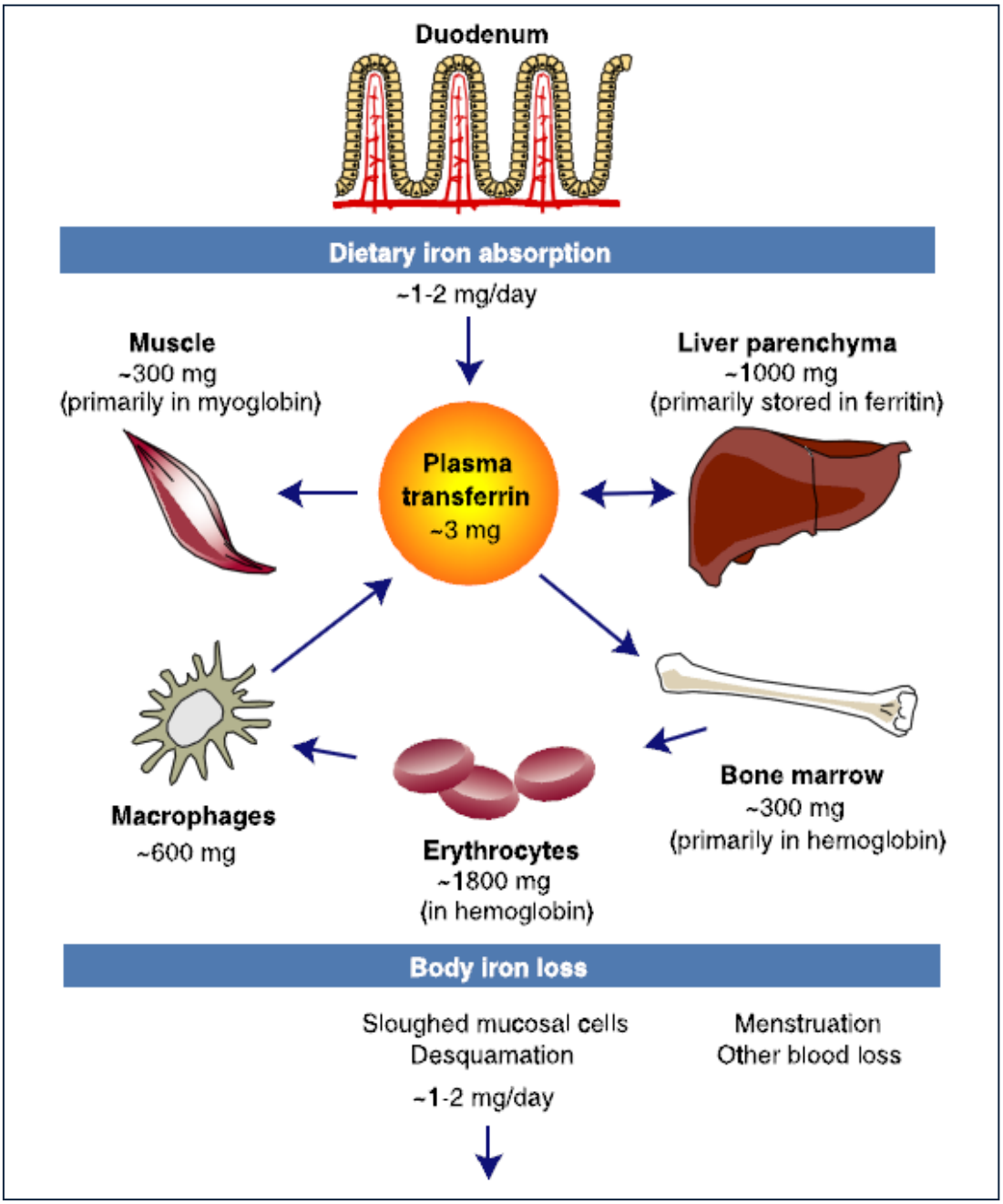


Figure 1.1 Iron distribution

The duodenum is the main site of dietary iron absorption to replace the iron lost through epithelial cell shedding and blood loss. To maintain iron homeostasis requires coordination between absorption, utilization and storage sites (Pantopoulos 2004, Papanikolaou and Pantopoulos 2005).

1.2.1. Iron absorption

There are two main forms of dietary iron: haem and non haem iron. Non-haem iron is found mainly in plant-based foods and accounts for about 90 – 95 % of total daily iron intake but its bioavailability is poor (1 - 10 %). Haem-bound iron which is found mainly in meats, comprises 5 - 10 % of dietary iron but due to its high bioavailability, it contributes to almost half of the daily iron absorption in western diets (Sharp & Srai 2007).

Dietary iron is predominantly absorbed in the duodenum and upper jejunum although the transport pathway for haem and non-haem iron differs across the apical membrane of enterocytes. Little is known about the mechanism of haem uptake. Recently, haem carrier protein 1 (HCP1) was identified as an intestinal haem transporter (Shayeghi et al. 2005) whose expression was regulated by iron deficiency and hypoxia. However, this was later demonstrated to mainly function as a proton-coupled folate transporter (Qiu et al. 2006). It is thought that once inside the cell, haem is degraded by haem oxygenase (HO-1) and the iron released enters a common intracellular pool merging with the non-haem iron transport pathway to be processed for subsequent release.

Non-haem iron exists primarily in the insoluble ferric (Fe^{3+}) state which must first be reduced to ferrous iron (Fe^{2+}) for transport across the epithelium. This is accredited to *duodenal cytochrome B* (DcytB), an endogenous ferric reductase found on the apical membrane of enterocytes (McKie et al. 2001). Dietary components such as ascorbic acid are also thought to contribute to this reduction process (Sharp and Srai 2007). Iron is transported across the apical membrane into the cell by *divalent metal*

transporter 1 (DMT1). The acidic environment provides a rich source of protons required for DMT1 co-transport of iron as well as other divalent metals such as Cu^{2+} (Gunshin et al. 1997, Tandy et al. 2000).

Once inside the cell, iron can either be stored as ferritin or transferred to the basal transporter *ferroportin* (FPN) for transport out of the cell and into the circulation (McKie et al. 2000). FPN releases ferrous iron which must be re-oxidised to its ferric state to be able to bind to circulating transferrin for ongoing transport. This oxidation at the basolateral surface is most likely performed by *hephaestin* (Hp) – a membrane-bound, copper-dependent ferroxidase (Chen et al. 2004, Vulpe et al. 1999).

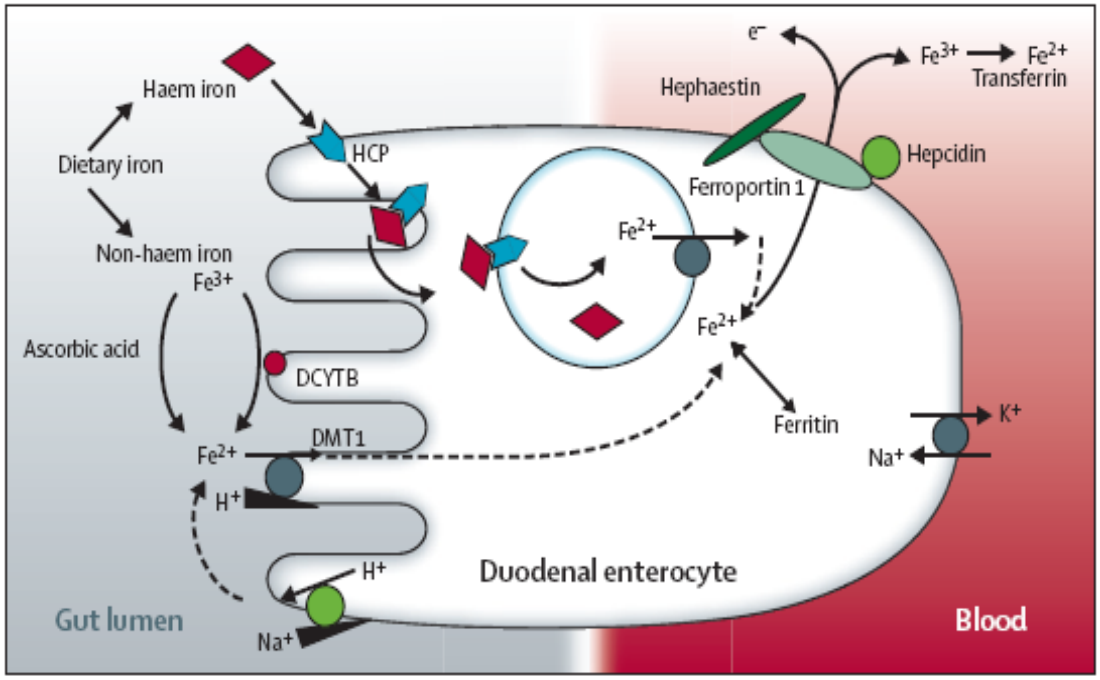


Figure 1.2 Intestinal non-haem iron absorption

Dietary non-haem iron is preferentially absorbed in the in the ferrous (Fe^{2+}) state. For non-haem iron this reduction is caused by dietary components e.g. ascorbic acid or endogenously by the ferriductase DcytB which is located on the brush border. Iron is transported through the apical membrane by DMT1 by a proton coupled mechanism. Inside, iron can bind to ferritin for storage, or be transported to the basolateral transporter FPN (ferroportin) for export. Iron is oxidised back to Fe^{3+} by hephaestin and bound to transferrin for re-distribution (Zimmermann and Hurrell 2007).

1.3. Proteins involved in iron transport

1.3.1. Duodenal cytochrome B (DcytB)

DcytB is a 286 amino acid-long ferric reductase located primarily at the intestinal brush-border membrane (McKie et al. 2001). Discovered in 2001 by subtractive cloning, the process gave way to finding other key regulators of iron metabolism including DMT1 and ferroportin. The location and close proximity of DcytB to DMT1

suggests its involvement in dietary iron absorption: reducing ferric iron to the more accessible ferrous form for DMT1 mediated cellular uptake (Wyman et al. 2008). Additionally, there is a quick induction of DcytB gene and protein expression in response to iron deficiency and hypoxia further supporting its functional role in iron metabolism (McKie et al. 2001). Surprisingly in DcytB knockout mice, there were no changes in iron stores and no observable signs of iron-deficiency. This suggests that DcytB is not needed for iron absorption in mice and has perhaps other physiological roles. However, it can be argued that the roles of DcytB may indeed differ between species as mice only express DcytB protein in erythroblasts whereas humans express it in many tissues (Su et al. 2006). Also, mice have less need for ferric reductase because they are capable of producing large amounts of ascorbic acid for iron reduction whereas humans cannot and are reliant on dietary vitamin C (McKie et al. 2001, Sharp and Srai 2007).

Progress is being made in finding other endogenous ferricreductases and the STEAP (six-transmembrane epithelial antigen of the prostate) family of reductases has recently been identified for its role in iron uptake in erythroid cells which, as well as iron, can also reduce copper (Ohgami et al. 2005).

1.3.2. Divalent metal transporter 1 (DMT1)

DMT1 is 561 amino acids long and contains 12 transmembrane spanning domains (Figure 1.3). It was initially called Nramp2 due its sequence similarity to Nramp1. To determine its function, a DMT1 mRNA construct was injected into Xenopus oocytes and screened for iron uptake activity (Gunshin et al. 1997). There was a substantial

increased uptake of iron compared to control and the name DCT1 (Divalent Cation Ttransporter 1) was suggested because Cd^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} were also shown to be transported (decreasing order of magnitude) (Garrick et al. 2003). It was also shown that the mechanism of DMT1 transport is proton-dependent, and therefore iron uptake is facilitated by the acidic environment of the proximal duodenum.

In parallel, its role in iron transport was confirmed, when a DMT1 gene mutation in microcytic mice (*mk*) and Belgrade rats (*b*) (Fleming et al. 1997, Fleming et al. 1998) caused a microcytic, hypochromic anaemia phenotype associated with defective iron uptake and gastrointestinal iron absorption. This highlighted DMT1 as a major transporter in intestinal iron uptake. Andrews (1999b) suggested the name be changed to DMT1 and this is now the most widely used name for this transporter (N. C. Andrews 1999b).

DMT1 has at least four different mRNA transcripts produced by alternative splicing at both the N-terminal and C-terminal regions (Hubert and Hentze 2002). Two variants are produced at the 5' end from alternative promoters with two different transcription sites classified as exon 1A and 1B (Figure 1.3). These are differentially expressed where exon 1B is ubiquitous, whereas 1A is tissue-specific with highest expression being in the duodenum and kidney (Hubert and Hentze 2002). It has been stipulated that these variants have different functional significance and their differing promoters also has potential regulatory significance (Garrick et al. 2003). Two further variants are created by alternative splicing at the 3' end either containing an iron response

element (IRE) (I) or non-IRE (II). Thus so far, four different isoforms have been identified resulting in different exons in mRNA and protein. Interestingly, in human intestinal Caco-2 cells it has been shown that variant DMT1B(I) can be regulated by iron whereas DMT1B(II) cannot, suggesting further IRE regulatory mechanisms may be in play. Moreover, iron has a regulatory effect on DMT1B(I) in mouse kidney cells but not in mouse duodenal cells suggesting iron-regulation of DMT1 may also be tissue-specific (Hubert and Hentze 2002).

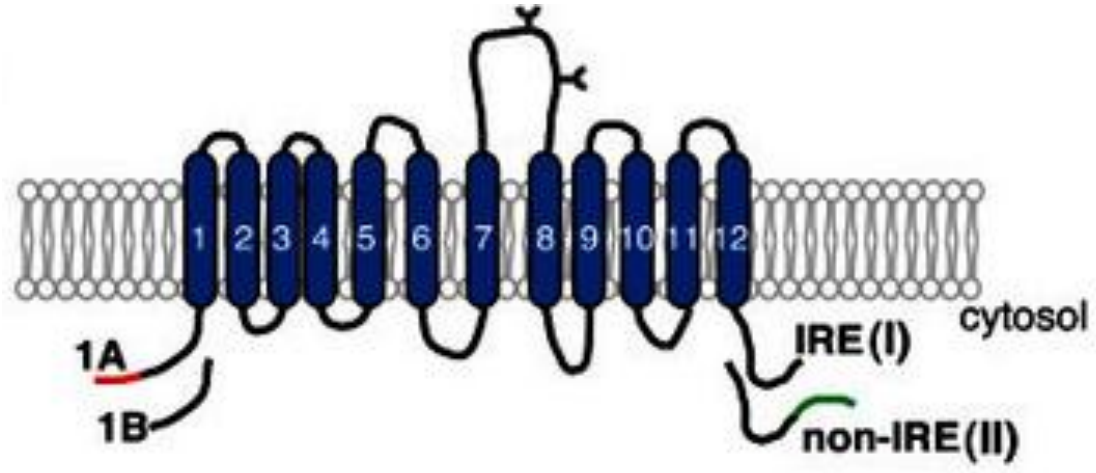


Figure 1.3 Model of DMT1 transporter

DMT1 has 12 transmembrane regions with N- and C- termini facing the cytosol and two potential glycosylation sites at the fourth extracellular loop. There are four isoforms: Exons 1A and 1B are produced by 2 different promoters at the N-terminal, whereas variants in the C-terminal are produced by alternative splicing with IRE (I) or without-IRE (II) (Yanatori et al. 2010).

1.3.3. Ferroportin (FPN)

Ferroportin (FPN) is the only known mammalian iron exporter. A product of the SLC40A1 gene, it encodes for a protein 571 amino acids in length with a predicted

mass of 62 kDa. FPN was discovered by three independent groups, by three different methods, each of whom gave it a different name, thus it can be called metal transporter 1 (MTP1), iron-regulated protein 1 (IREG1) and ferroportin-1 (FPN1) respectively (Abboud and Haile 2000, McKie et al. 2000, Donovan et al. 2000). Its importance in iron homeostasis has been well documented. In cell culture models, over-expression of FPN caused intracellular iron depletion and tissue-specific activity was demonstrated by iron deficiency upregulating FPN expression in mouse duodenum but down-regulating expression in liver (Abboud and Haile 2000). Using Xenopus oocytes, FPN was shown to function as an iron exporter, stimulating iron efflux at the basolateral surface of polarised epithelial cells, capable of exporting cellular iron into the circulation (Donovan et al. 2000, McKie et al. 2000). Insight was given into the FPN structure when it was shown that the 5'UTR of FPN contained a functional IRE. However, in iron deficient conditions, this IRE would cause translation repression after IRP binding whereas FPN mRNA and protein increased in such conditions, making it unclear whether the IRE/IRP complex affects translation or stability of FPN (McKie et al. 2000).

FPN has more than 90% homology between humans, mice and rats, making it a highly conserved protein inter-species. This conservation suggests FPN has a fundamental role in iron homeostasis. FPN is the only known iron exporter in vertebrates. Complete inactivation of FPN in mice and zebrafish caused embryonic lethality indicating its essential role in early development (Donovan et al. 2005). Mice that were FPN-deficient after birth showed iron accumulation in enterocytes, hepatocytes and macrophages, stressing a key role of FPN for iron release within these cell types.

Intestine-specific inactivation caused duodenal iron accumulation in this tissue only, confirming that it is critical for iron absorption (Donovan et al. 2005). This highlights FPN as one of the most important proteins in iron homeostasis.

However there is still a lot unknown about FPN. Little work has been carried out on the FPN structure to identify how it carries out transmembrane iron transport and there is no general consensus on its topology. Earlier studies proposed it to have ten transmembrane domains (Donovan et al. 2000, McKie et al. 2000) but more recent topology software has predicted 12 transmembrane domains (Liu et al. 2005). The positions of N- and C- termini are also under debate with some suggesting both termini to be intracellular (Liu et al. 2005) whilst others depicting an intracellular N-terminal and an extracellular C- terminal (Devalia et al. 2002). Another structural controversy is whether FPN is a monomer or a multimer. Coimmunoprecipitation of FPN with different epitope tags and detection of FPN with a larger mass than the monomer, indicate that FPN functions as a multimer (De Domenico et al. 2005). However in similar epitope-tagged studies, there was a lack of coimmunoprecipitation and co-localisation of mutant with wild-type FPN arguing against FPN hetero-oligomers and *in vivo* studies suggested FPN as a monomer (Goncalves et al. 2006, Schimanski et al. 2005, Schimanski et al. 2008). A more recent study has shown that FPN is a monomer. Using multiple techniques including size exclusion chromatography and UV absorption spectroscopy analysis, purified, detergent-solubilized human FPN was observed as a single peak. Furthermore, SDS-PAGE analysis revealed a single predominant band with a molecular mass of approximately 60 kDa in agreement with its predicted weight (Rice et al. 2009). Although it was

noted that a transient dimerisation/multimerisation event could not be completely ruled out. Expression of FPN was observed at the basolateral membrane of polarised epithelial cells but not at the apical surface. To address the issue of N-/C- termini location, confocal microscopy utilising fluorescent antibodies, strongly supported cytosolic location for both termini and a 12 transmembrane domain topology similar to (Liu et al. 2005) was suggested (Rice et al. 2009).

The 5'UTR of FPN mRNA contains a putative IRE that could indicate translational regulation by iron regulatory proteins (IRPs) in a similar way to other IRE-regulated genes (e.g. ferritin) (Cianetti et al. 2010). Experiments with luciferase reporter gene assays have shown that this 5'IRE was responsive to iron in HepG2 (hepatocyte), Caco-2 (enterocyte) and U937 (macrophage) cells (Lymboussaki et al. 2003). Recently a variant of FPN, FPN1B, has been identified which lacks the IRE by using an alternative upstream promoter allowing it to evade the IRE/IRP system (Zhang et al. 2009) thus it could possibly undergo post-translational regulation instead. FPN1B is more specific to duodenal epithelial and erythroid precursor cells suggesting a tissue-specific regulatory mechanism. Iron regulation of FPN appears to be highly complex with many levels of possible regulation, both transcriptional and post-transcriptional but as the only known significant iron exporter in tissues involved in iron absorption, storage and recycling, its role in iron homeostasis is clearly indispensable.

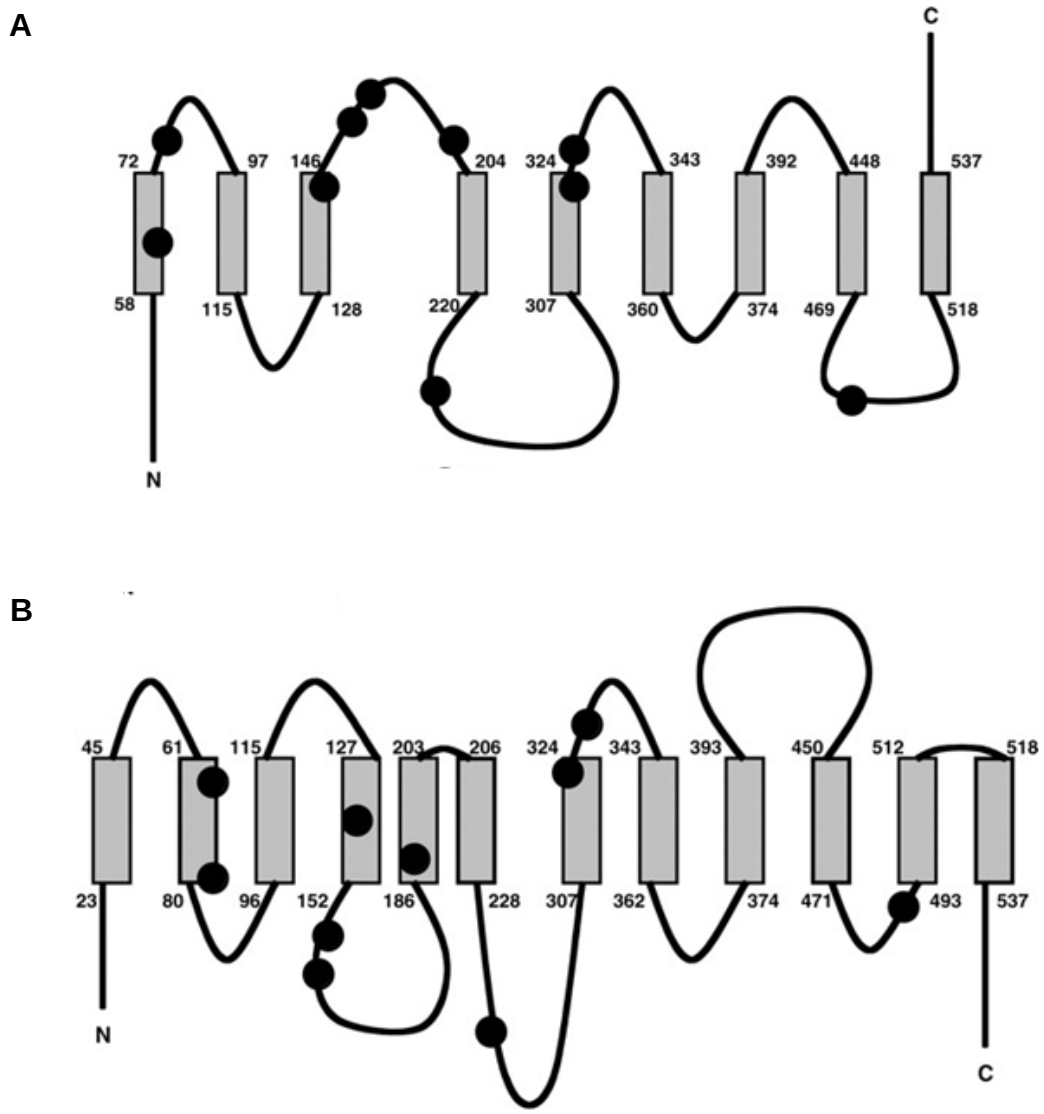


Figure 1.4 Predicted models of FPN transporter

Topology of FPN protein, modified from two alternative models. **(A)** 9 transmembrane helices proposed by (Devalia et al. 2002). **(B)** 12 transmembrane helices by (Liu et al. 2005). Adapted from (Wessling-Resnick 2006). Black filled circles represent known possible human mutations. N- and C-termini denoted by N and C respectively. Top of each diagram represents extracellular space and bottom is cytoplasm.

1.3.4. Hephaestin

Hephaestin is a multi-copper oxidase required for basolateral oxidization of iron from enterocytes, to allow iron binding to circulating transferrin and is therefore categorized as a ferroxidase (Vulpe et al. 1999). Encoded by the *HEPH* gene, the product is a transmembrane-bound protein, which is 50% identical at the protein level to serum oxidase ceruloplasmin. Both contain iron and copper binding sites but where their function may be similar, their location is not. Ceruloplasmin is expressed mainly in liver and is plasma-soluble whereas hephaestin is highly expressed in the intestine and anchored to the plasma membrane (Chen et al. 2004, Vulpe et al. 1999).

A spontaneous mutation in HEPH has been observed in sex-linked anaemia (sla) mice through gene-mapping studies. A deletion of 582 bases in the mRNA of the gene produces a shortened protein missing 194 amino acids. This mutation results in a failure of hephaestin to localise to the basolateral membrane of the intestine, causing functional defects (Kuo et al. 2004). Mice exhibit intestinal iron accumulation and systemic iron deficiency suggesting the important role of hephaestin in basolateral iron export (Chen et al. 2004, Vulpe et al. 1999).

Unlike the other proteins of iron transport, hephaestin expression does not appear to be significantly affected by local cellular iron levels (Chen et al. 2003). However, hephaestin mRNA was increased in the duodenum of iron-deficient mice thereby possibly suggesting that hephaestin expression is more responsive to systemic iron levels than that of iron levels in enterocytes (Chen et al. 2003). It is also important to remember that hephaestin requires copper for structural stability and enzymatic activity and therefore levels of copper affect hephaestin expression. Indeed hephaestin

protein and ferroxidase activity have been shown to decrease in copper-deficient mice and rats respectively (Chen et al. 2006, Reeves et al. 2005) confirming that copper is required for the proper synthesis and functioning of hephaestin.

1.3.5. Ferritin

Ferritin is the primary iron storage protein found ubiquitously in tissues. Ferritin is a 450 kDa protein comprising of 24 apoferritin monomers that come together to form a hollow spherical particle (Harrison and Arosio 1996) (Figure 1.5). This sphere is capable of binding up to 4500 ferric atoms, making it a very effective iron delivery system (Wang et al. 2010). The ferritin molecule consists of different ratios of two subunits termed L (light) and H (heavy). H refers to the fact it is the heavier of the two subunits weighing 21 kDa or to its original isolation from the human heart, which is rich in the H subunit. L is the lighter subunit weighing 19 kDa and is the predominant form in liver (Wang et al. 2010, Torti and Torti 2002). Most tissue ferritins are a mixture of the subunits, with the ratio depending on tissue type, developmental stage, inflammatory condition, amongst other stimuli (Torti and Torti 2002). H subunit has enzymatic ferroxidase activity which allows internalisation and sequestration of iron in the ferritin core (Lawson et al. 1989).

Ferritin can be regulated post-transcriptionally by the IRE/IRP system which responds to intracellular iron levels. A more in-depth discussion of the IRE/IRP system can be found in the next section (1.4.1). Ferritin mRNA contains IRE in its 5'UTR (Hentze et al. 1987), which bind IRPs when iron is low, inhibiting translation so less of the protein is synthesized when it is not required (Torti and Torti 2002).

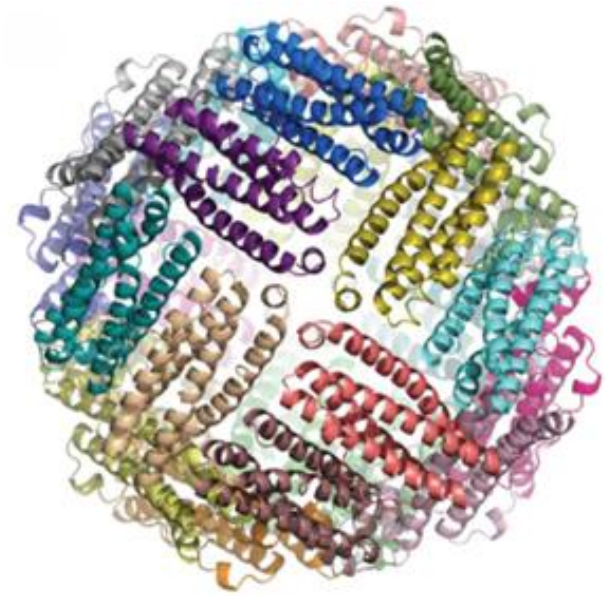


Figure 1.5 Tertiary structure of ferritin multimer

Iron storage protein ferritin consists of 24 monomers of heavy and light subunits to form a central spherical cage which can hold up to 4500 ferric atoms in a non-toxic form (Marchetti et al. 2009).

1.4. Regulation of iron homeostasis

1.4.1. Cellular control: IRE/IRP system

Cellular iron levels are maintained by coordinating iron uptake, utilisation and storage. It is thought that the main regulation of cellular iron levels occurs post-transcriptionally to allow for quicker changes in protein expression by changing the rate of mRNA synthesis. In iron metabolism, this involves iron-response element (IRE) / iron regulatory protein (IRP) interaction.

IREs are conserved hairpin structures of 25 – 30 base pairs that are located on 3'- or 5'- UTR (untranslated regions) of mRNA that encode for proteins of iron metabolism.

The cellular liable iron pool regulates binding of IRP1 and IRP2 to these elements to optimise iron availability.

IRP1 is an iron-cluster containing protein which has a dual function alternating between an IRP and an active cytosolic aconitase. During normal or high iron levels, IRP1 has a 4Fe-4S cluster ligated to its active site preventing it from binding to IRE and functions as an aconitase. However, when iron levels are low, the iron-sulphur cluster dissembles and IRP1 assumes an open configuration that can bind to the IRE hairpin structure. Conversely IRP2 is produced *de novo* during iron depletion but targeted for rapidly degradation in iron-replete cells (Iwai et al. 1998).

The location of IRE results in differential effects. IRPs binding to 5'-IRE prevent ribosomal binding to target mRNA thereby inhibiting translation. Whereas IRPs binding to 3'-IRE prevent endonuclease degradation resulting in mRNA stabilisation and increased protein production (Binder et al. 1994).

DMT1 and transferrin receptor 1 (TfR1) mRNA both have IREs at the 3'UTR (DMT1 has a single IRE while TfR1 has five IREs) whereas FPN and ferritin have an IRE at the 5'-UTR. Therefore in low iron conditions, IRPs stabilise DMT1 mRNA increasing protein synthesis to promote cellular iron import; FPN and ferritin translation are repressed. Conversely, in high iron conditions, FPN and ferritin translation proceeds for export and storage of excess iron respectively and DMT1 mRNA is destabilised to stop further cellular uptake of iron (Torti and Torti 2002).

IRP2 is a slightly larger protein than IRP1 (105 kDa and 87 kDa respectively) due to an extra 73 amino acids but despite being 60 – 70% homologous with IRP1, IRP2 does not possess any aconitase activity and its primary function appears to be controlling translation of IRE-containing mRNAs. There is also some tissue specificity as IRP1 activity in rodents is abundant in liver, intestine and kidney, while IRP2 has highest activity in intestine and brain (Henderson et al. 1993). IRP2 appears to be the better iron sensor as it is able to complement IRP1 function in IRP1 knockout mice which did not show any overt signs of abnormality (LaVaute et al. 2001). IRP2 knockout mice, however, exhibited misregulated iron metabolism in the intestinal mucosa and the central nervous system suggesting that IRP2 is more important in regulating iron metabolism as IRP1 cannot compensate for the loss of function. Knockout of both IRP1 and IRP2 caused embryonic lethality (Smith et al. 2006) demonstrating that the IRE/IRP system is essential for life.

The IRE/IRP system regulates a number of iron-metabolism proteins which allows for homeostatic control to occur at a cellular level.

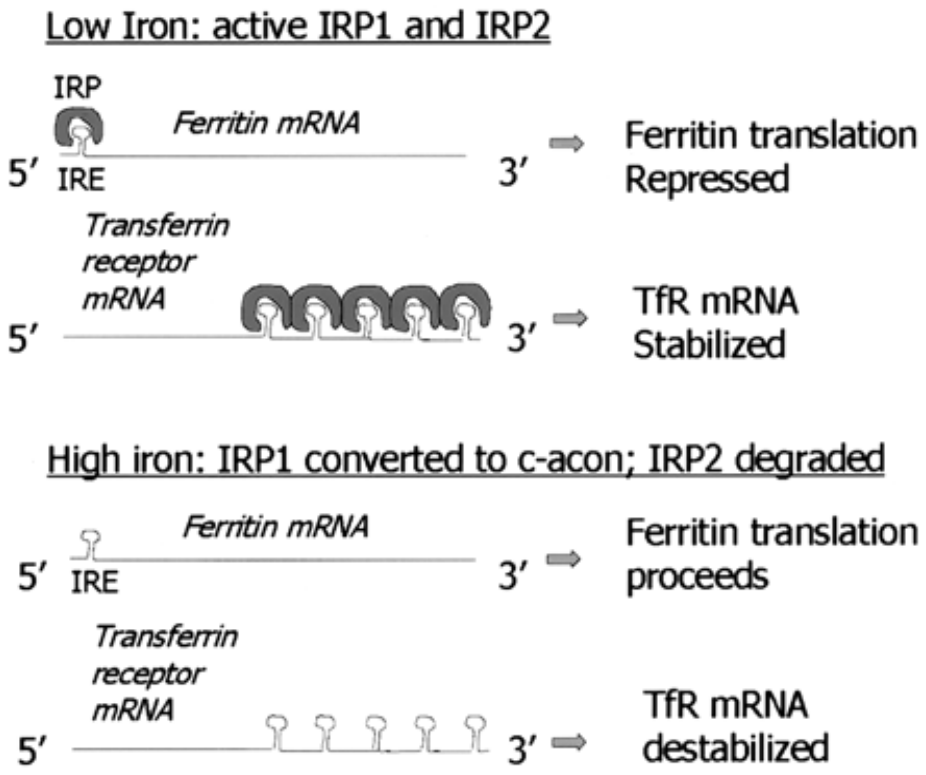


Figure 1.6 IRE/IRP regulation of ferritin and TfR

Low iron causes binding of IRP1 and IRP2 to IRE in ferritin 5'-UTR resulting in repressed ferritin translation. However, if the IRE is in 3'-UTR like TfR, then IRP binding stabilizes mRNA and increases translation. During high levels of iron, IRP1 is converted to aconitase with no IRP activity and IRP2 is rapidly degraded resulting in increased translation of ferrtin for iron storage whilst less TfR is synthesized. IRP: iron regulatory protein 1; IRP2: iron regulatory protein 2; IRE: iron responsive element; TfR: transferrin receptor (Torti and Torti 2002).

1.4.2. Systemic control: Hepcidin

The discovery of hepcidin and its connection to iron came about in many ways. A novel antimicrobial peptide, highly expressed in the liver, was isolated by Krause (Krause et al. 2000) and named LEAP-1 (liver expressed antimicrobial peptide). Around the same time, (Park et al. 2001) isolated the same peptide from human urine

and named it hepcidin (hepatic origin and it being a bactericidal protein). In humans, hepcidin in encoded by the HAMP gene synthesizing an 84 amino acid protein known as a preprohepcidin. This is cleaved to prohepcidin which is 60 amino and further processed to give the bioactive mature 25 amino acids hepcidin. Low levels of shorter hepcidin peptides (20 and 22 amino acids) can also be detected but they possess little activity and are likely to be the degradation products of the mature form (Nemeth et al. 2006).

A lot of the information we know about hepcidin comes from *in vitro* studies and mouse models. The role of hepcidin in iron metabolism was first established in hepcidin gene-knockout mice which developed severe iron overload with iron depositions in liver, pancreas and heart (Nicolas et al. 2001). Conversely, mice over-expressing hepcidin were born with severe iron deficiency and died soon after birth (Nicolas et al. 2002). In adult rats switched from an iron-replete diet to an iron-deficient diet, hepcidin expression was shown to inversely correlate with iron absorption and duodenal iron transporters (Frazer et al. 2002). These finding are all consistent with hepcidin acting as a negative regulator of iron absorption. This key connection was underlined in humans when it was discovered that patients with juvenile haemochromatosis had inactivating hepcidin mutations causing severe iron overload (Roetto et al. 2003) confirming the importance of hepcidin in iron homeostasis.

The only known molecular target for hepcidin is FPN (Nemeth et al. 2004b). FPN is the only known exporter responsible for exporting iron from intestinal enterocytes,

macrophages, hepatocytes and placenta to the circulation. Hepcidin acts by directly binding to FPN, causing its internalisation and subsequent degradation in lysosomes (Nemeth et al. 2004b). This loss of FPN from cell surfaces prevents cellular iron export which creates a negative feedback cycle to inhibit further hepcidin release. Therefore when iron levels are high, hepcidin is produced by the liver and targets FPN on the basolateral membrane of enterocytes to block release of dietary iron to transferrin thus reducing plasma iron levels. In macrophages, degradation of FPN causes iron trapping characteristic of iron-containing macrophages in inflammation when circulating levels of hepcidin are high (Nemeth and Ganz 2006). When iron returns to normal levels (or low), hepcidin production is inhibited, allowing FPN re-synthesis and iron transport and recycling to resume (Figure 1.7).

FPN1B (-IRE) is the more abundant isoform in the intestine and since it is not regulated by the IRE/IRP system, it is more likely that FPN1B is regulated at a post-translational level by hepcidin. Hepcidin may also indirectly affect the IRE/IRP system. By inhibiting iron efflux, intracellular iron content is increased which may affect the expression of iron importer DMT1+IRE, transferrin and possibly other pathways. However this interaction of hepcidin-FPN with the IRE/IRP system is not well understood and yet to be confirmed (Nemeth and Ganz 2006).

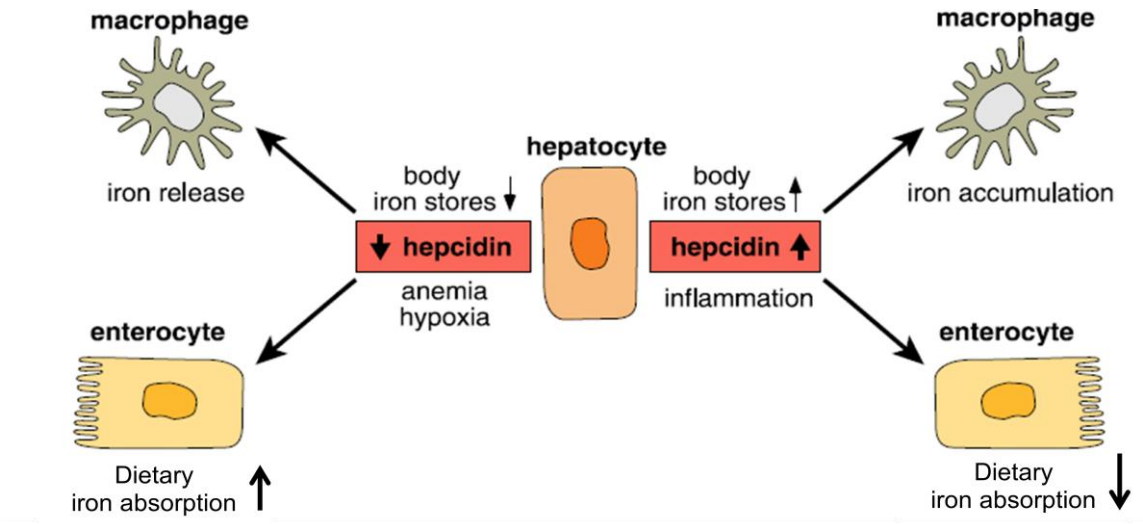


Figure 1.7 Regulatory functions of hepcidin

Reduced plasma hepcidin levels, as a result of reduced body iron stores promotes dietary iron absorption in the small intestine and increased iron release from macrophages. An increase in hepcidin levels stimulated by high body iron or inflammation inhibits dietary iron absorption and iron release from macrophages (Redrawn from Papanikolaou and Pantopoulos 2005).

1.4.3. Other regulators of iron: inflammation; erythropoiesis; hypoxia

An increase of iron causes increased hepcidin production but the mechanism by which this occurs is not known. Hepcidin is produced by the liver but it is not clear if this is where iron levels are sensed. Iron loading of primary human and mouse hepatocytes *in vitro* does not affect hepcidin mRNA suggesting that other cell types may be needed for iron sensing (Pigeon et al. 2001, Nemeth et al. 2003). The HAMP gene encoding for hepcidin does not contain any IRE ruling out IRP binding as a regulatory mechanism and suggests iron regulation of hepcidin by other signalling pathways instead. Patients with hereditary haemochromatosis (HH) suffer from iron overload due to defective hepcidin regulation. In both patients and mice models of the disease, mutations have been found in HFE (haemochromatosis protein), transferrin receptor 2

(TfR2), and hemojuvelin (HJV), strongly suggesting that these proteins have a role in iron regulation of hepcidin (Bridle et al. 2003, Kawabata et al. 2005, Niederkofler et al. 2005, Pissia et al. 2004). If and how these pathways interact to regulate hepcidin remains elusive.

During infection and inflammation, hepcidin levels are increased. This is a host-defence mechanism aiming to reduce available iron to invading organisms. This effect is mediated by inflammatory cytokines, namely interleukin-6 (IL-6). Healthy human volunteers that were infused with recombinant human IL-6 showed a rapid increase in hepcidin expression (as measured by urinary excretion) matched with a significant decrease of serum iron and transferrin saturation. IL-6 KO-mice failed to produce hepcidin mRNA in response to turpentine-induced inflammation (Nemeth et al. 2004a). These parallel results show that IL-6 is an important regulator of hepcidin during inflammation. Recently other cytokines have also been indicated to play a role but findings have been controversial. In mice with targeted IL-6 gene disruption (IL-6-/-) IL-1α and IL-1β were both shown to be potent hepcidin inducers (Lee et al. 2005) but no affect was observed in primary human hepatocytes (Nemeth et al. 2003). In primary rat hepatocytes TNFα exhibited stimulative effects on hepcidin mRNA expression (Dzikaite et al. 2006) whereas it had a suppressive hepcidin effect in human hepatocytes *in vitro* (Nemeth et al. 2004a). This suggests that there may be variations in the effects of inflammatory cytokines between species (Nemeth and Ganz 2006).

Erythropoiesis is a hepcidin suppressor, capable of overriding systemic iron status (Figure 1.8). During anaemia or hypoxia, the body responds by producing the protein erythropoietin (Epo) from the kidneys to stimulate erythroid differentiation at the bone marrow. This process decreases hepcidin levels which allow more dietary iron to be absorbed and mobilisation of iron from macrophages and hepatocytes to sustain erythropoiesis.

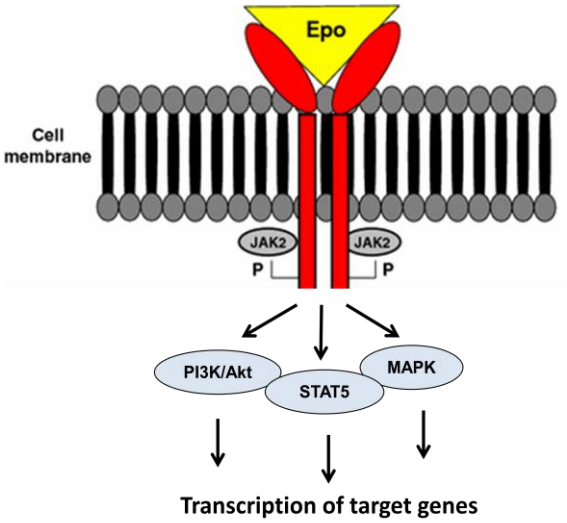


Figure 1.8 Simplified schematic of Epo signalling

Binding of Epo to two adjoining receptors leads to their homodimerization and phosphorylation of JAK2. This in turn phosphorylates tyrosine residues on the cytoplasmatic tail of Epo-R producing docking sites for various downstream signalling molecules such as PI3K, STAT5 and MAPK. These molecules eventually migrate to the nucleus to increase target gene expression. (Adapted and redrawn from Marzo et al. 2008).

The exact mechanism of how Epo regulates hepcidin is not clear. Hepatocytes express Epo receptors (Epo-R). Through *in vitro* work in HepG2 human hepatocyte cell line, it has been proposed that Epo binds directly to hepatic Epo-R decreasing transcriptional

factor C/EBPα (CCAAT/enhancer binding protein α) binding to the hepcidin promoter, thereby suppressing hepcidin expression (Pinto et al. 2008). However studies in mice suggest that Epo suppression of hepcidin is caused by downregulation of STAT3 and SMAD phosphorylation (Huang et al. 2009) whilst other studies suggest the effect of Epo is mainly indirect and an intermediary bone marrow response is required (Zhang and Enns 2009). It is not yet clear which aspect has most influence on Epo regulation of hepcidin.

Hypoxia is a physiological stimulus of eythropoiesis and hypoxia inducible factors (HIFs) play an important role in this process. HIFs are transcription factors comprised of two subunits: an oxygen sensitive α subunit (HIF1α/HIF2α) and a constitutively expressed β subunit. Although HIF1 and HIF2 share many common targets, they also have separate physiological functions and HIF2 appears to regulate Epo production in adult life. In normoxic conditions HIF1α is modified by prolyl hydroxlases (PHDs) and is subjected to proteosomal degradation. During hypoxia however, PHD activity is inhibited, allowing HIF1α accumulation and translocation to the nucleus whereupon it dimerises with the β subunit and binds to hepcidin promoter suppressing hepcidin expression (Patnaik and Tefferi 2009, Peyssonnaux et al. 2007). This increases iron uptake to meet erythropoietic demands. Human hepcidin promoter contains several HIF binding sites but their exact role has not been tested so whether HIF binds directly to the hepcidin promoter to inhibit its activity remains controversial.

Thalassemia patients display low levels of hepcidin even with systemic iron overload indicating that signals for hepcidin suppression dominate over signals of systemic iron

and that Epo signaling may be the most potent inhibitor of hepcidin expression (Papanikolaou et al. 2005). Interestingly, pro-inflammatory cytokine IL-6 failed to induce hepcidin activation in the presence of HIF stabalisation (Peyssonnaux et al. 2007). This demonstrates a link between inflammation, hypoxia and erythropoiesis although the precise mechanism by which they down-regulate hepcidin has yet to be defined.

1.4.4. Dietary influence on non-haem iron bioavailability

Haem iron absorption is relatively unchanged by meal compositions. However, absorption of non-haem iron, which is the more abundant form, can be substantially altered by co-consumption of certain dietary factors (

Figure **1.9**). Vitamin C is a powerful enhancer of non-haem iron absorption; the primary mechanism thought to be by its ability to reduce Fe^{3+} to Fe^{2+}, which is the form taken up by mucosal cells (Plug et al. 1984) as stomach contents enter the duodenum. There are many studies showing a clear dose-dependent effect of vitamin C increasing non-haem iron absorption further supporting its promoter activity (review of 24 studies by (Bendich and Cohen 1990).

Polyphenols are among the most significant dietary inhibitors of non-haem iron absorption. Their widespread distribution in food means they are likely to be present in most meals and allow interaction with dietary intake of iron. Polyphenols are known to be effective iron chelators (Afanas'ev et al. 1989) which is one of the ways they may affect non-haem iron bioavailability.

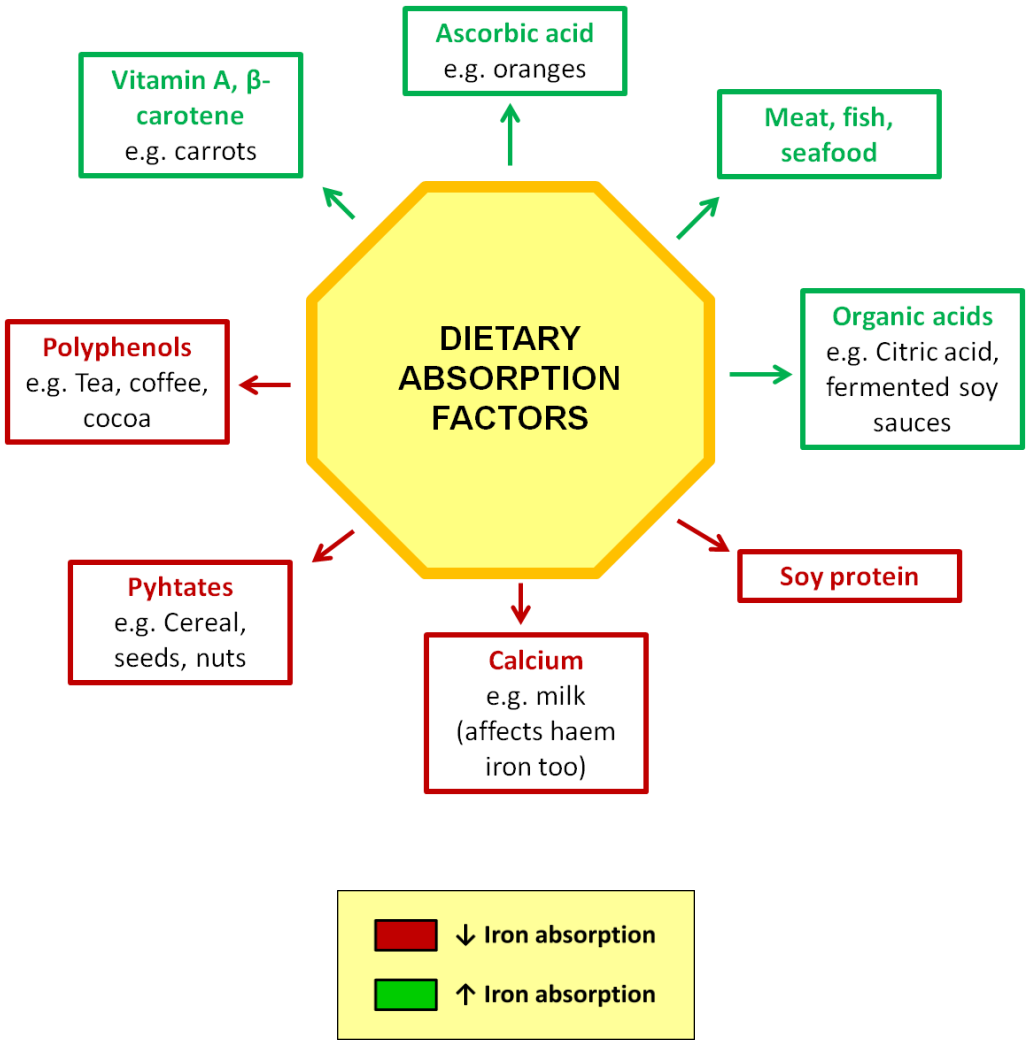


Figure 1.9 Dietary factors affecting absorption of non-haem iron

1.5. Polyphenols

Polyphenols are a complex group of chemical molecules derived from plants. Found naturally in fruits, vegetables and flowers, the beneficial health effects of plants were known long before polyphenols and in particular, flavonoids, were isolated as the effective compound.

The Hungarian scientist Albert Szent-Gyorgyi was the first to identify the structure of ascorbic acid (commonly known as vitamin C) and its isolation resulted in the treatment of scurvy (caused by vitamin C deficiency). A fellow colleague had a patient suffering from subcutaneous capillary bleedings and it was suggested that vitamin C might help but as sufficient amounts were not yet available, an impure substance containing a mixture of vitamin C plus other compounds, was given and achieved rapid success. When pure vitamin C was later tested on its own, the same results could not be obtained. This suggested to Szent-Gyorgyi that another compound must be contributing to the therapeutic effect and going back to the impure substance, he suspected a group of plant-based compound with polyphenol structure as a key factor. Based on his experience with vitamin C and his earlier observation that the therapeutic effects of a mixed substance was more beneficial than vitamin C alone, he suggested that these polyphenols might interact with vitamin C to repair damaged tissues by increasing resistance and maintaining integrity of capillary walls. Due to some of the compounds possessing vitamin-like properties, he called the polyphenols ‘vitamin P’ (Szent-Györgyi 1937). This name was later dropped as there was not substantial evidence to show that polyphenols were essential like vitamins. Szent-Gyorgyi proposed that polyphenols possess great biological activity and had the

potential of being effective therapeutic agents for a range of diseases. His work on vitamin C and 'vitamin P' was recognized in 1937 earning him a Nobel Prize in Medicine. Szent-Gyorgyi had said flavonoids "represent one of the most exciting, broad, and hopeful fields of biological inquiry" (Gabor 1988) and indeed this has inspired decades of investigative research in to polyphenols.

Polyphenols are essential for plant physiology, contributing to growth, morphology and protection as anti-feedants (unpalatable to predatory animals). Many polyphenols act as dyes, responsible for giving flowers their attractive colour and thus can also affect pollination by affecting insect feeding (Hedin and Waage 1986). Polyphenol structure can vary greatly and further sub-classing depends on the carbon skeleton. Of plant polyphenols, flavonoids represent the most widely distributed group but with over 6000 members (Harborne and Williams 2000), most experiments in the field focus on a much smaller number which are relevant from a dietary viewpoint.

1.6. Flavonoids

Flavonoids are low-molecular-weight polyphenol substances generally composed of a three-ring structure (Figure 1.10). They are formed from combining derivatives of shikimic acid pathway and acetic acid (reviewed by (Aherne and O'Brien 2002). The basic flavonoid structure contains 2 benzene rings (A and B), joined by an oxygen-containing pyrene ring (C). Variations in ring C and the substitution patterns available for rings A and B allow a variety of subgroups to exist. Classification depends on the extent of oxidation of the C ring. Their biological properties depend on their chemical

structure and degree of modification by additional moieties. Flavonoids are mostly present as glycosides, that is they have sugar moieties attached which increases their polarity, and lacking this, they are called aglycone. Common sugars are D-glucose and L-rhamnose with the preferred site of glycosylation being the C-3 and C-7 positions.

Flavonoids are present in most edible fruits and vegetables, but the type of plants they are found in has variations in different flavonoid content. Flavanols can be found in cocoa and related products; flavanones in citrus foods; flavones in green leafy spices such as parsley and capsicum pepper; isoflavones are found in soy foods, anthocyanidins in berries, and flavonols are found in almost all foods (Lakhanpal and Rai, 2007).

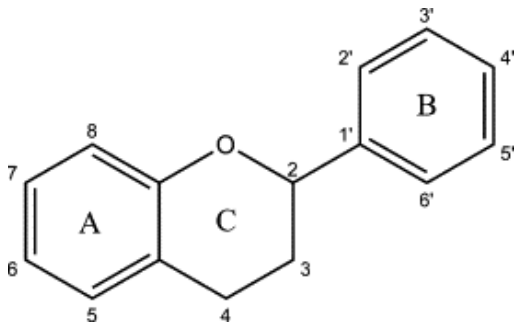


Figure 1.10 Basic structure and numbering system of flavonoids

Of the flavonoids, flavonols are the most common compounds, present in almost all plants. Quercetin is the main flavonol present in our diet. Its dietary abundance, along with its interesting chemical and biological properties, has made quercetin one of the most extensively studied flavonoids.

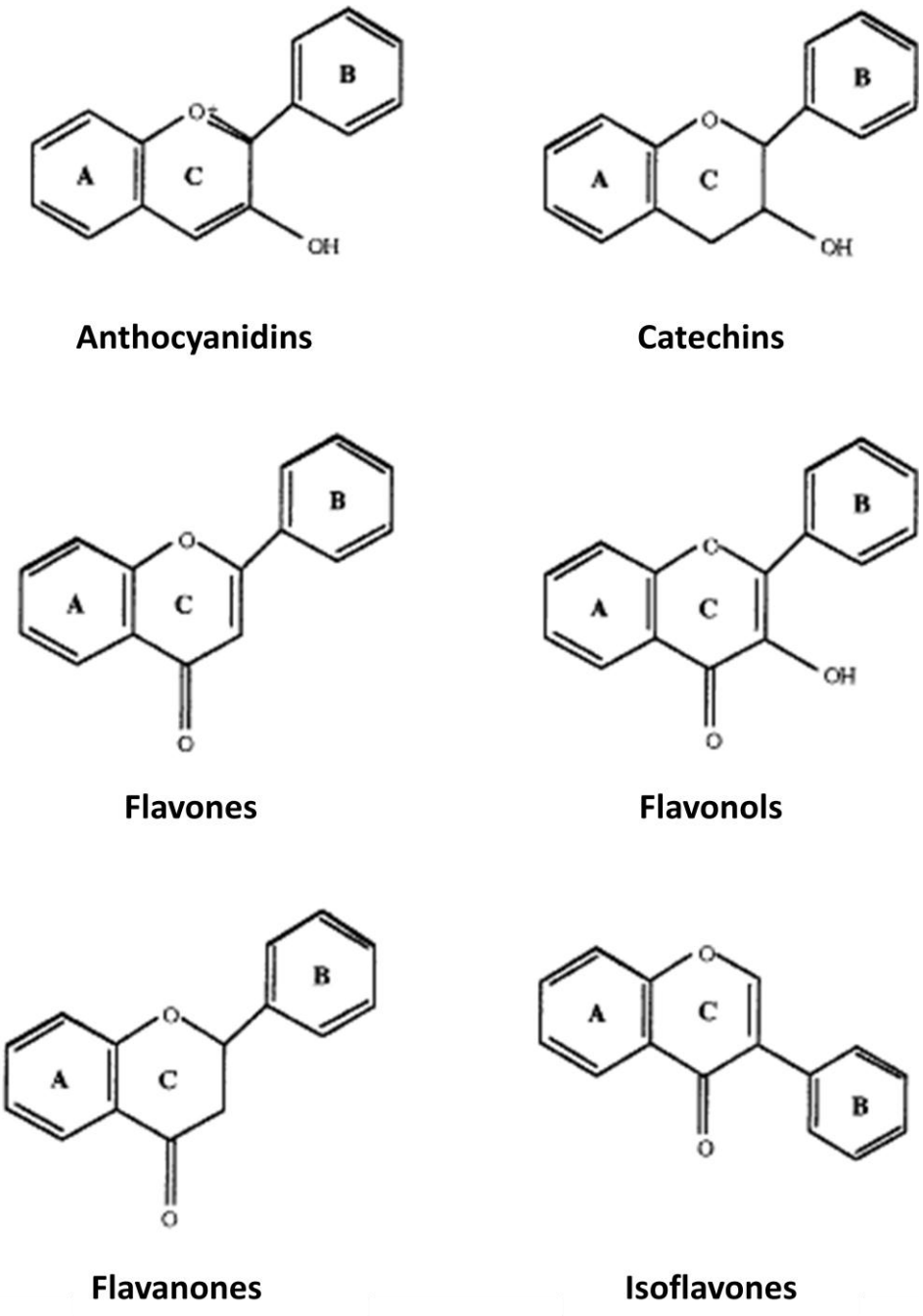


Figure 1.11 Major classes of food flavonoids

The structural classification of a flavonoid is based on the degree of hydroxylation of the C-ring. Adapted from Aherne and O'Brien 2002.

Quercetin is the predominant dietary flavonoid. In plants it occurs mostly in the glysosidic form where glycosylation can occur at any of the hydroxyl group to yield a sugar. Common sugars include glucose, galactose and rutinose which bind to aglycone quercetin at the 3-poistion (3-O-glycoside) to form rutin (Figure 1.13). The major glycosilation targets of quercetin appear to be the 3-, 7- and 4'-hydroxyl groups (Rice-Evans et al. 1996).

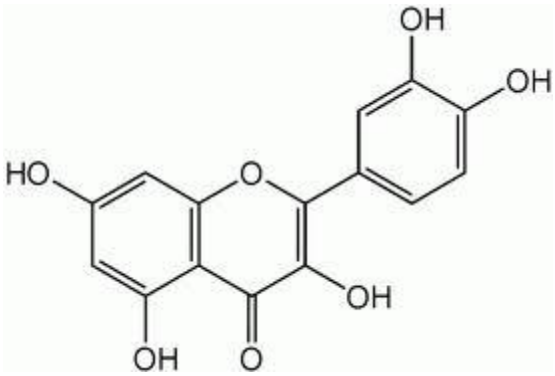


Figure 1.12 Structure of quercetin

Belonging to the flavonol subclass, quercetin is one of the most abundant flavonoids in the diet.

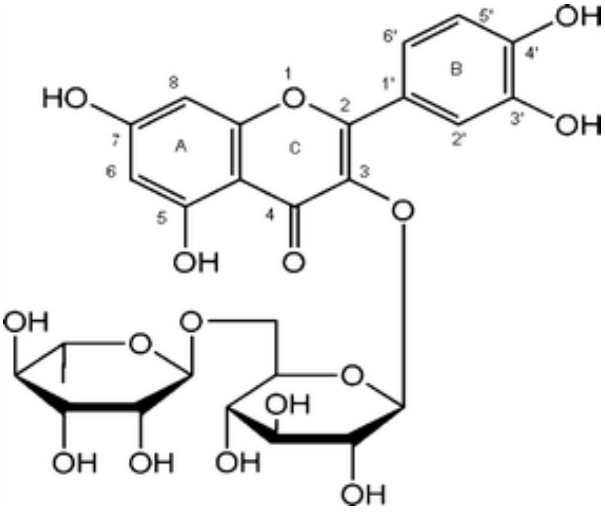


Figure 1.13 Structure of rutin

(quercetin-3-O-rutinoside).

Quercetin is present in many different glycosidic forms with rutin being one of the more common forms found (Erlund 2004). The 3-OH group appears to be a favoured glycosylation site.

Flavonoids are provided by the diet and to understand their biological effects, reliable data on flavonoid content within foods is needed. Substantial effort has been made in this regard, with the development of analytical methods such as the highly sensitive and selective high performance liquid chromatography (HPLC) being the method of choice. In a study by Hertog et al. (1992) five flavonoids were quantified in various commonly consumed fruits and vegetables. Flavonoid-glycosides were extracted and hydrolysed to their aglycone form, then separated and quantified by HPLC and UV detection. Table 1.1 shows quercetin content in a number of foods. Using this method, the United States Department of Agriculture has since composed a more extensive list of quercetin content in foodstuff.

Table 1.1 Quercetin content of various vegetables, fruits and beverages

Vegetables	Quercetin (mg/kg)	Fruits	Quercetin (mg/kg)	Beverages	Quercetin (mg/L)
Onion	340–347	Apple	20–36	Black tea	14–17
Kale	110–120	Black grape	15–37	Green tea	13-24
Cherry tomato	63	White grape	2–12	Tomato juice	13
Broccoli	30–37	Cherry	10–15	Red wine	8.3
Lettuce	14–79	Plum	9–15		
		Strawberry	6–8.6		

Queretin content as measured by HPLC methods after hydrolysis of glycosides. Only edible portions were used where appropriate (Hertog et al. 1992, Hertog et al. 1993, Hollman and Arts 2000).

1.6.1. Dietary intake

Currently there is no accurate information available on dietary intake of polyphenols, only estimations. This is because there is high variability in the types of food consumed, individual eating habits and processing of food affecting polyphenol content. Therefore quercetin sources differ greatly between and even within countries. In the Seven Countries Study, which started in the 1960s, Hertog et al calculated flavonol intakes from different populations and reported that tea was the predominant source of quercetin in Dutch and Japanese diets. Red wine was the greatest source of quercetin in Italy, whilst in the United States, Finland, and Greece, onions and apples were the main source (Hertog et al. 1995). Although onions may not be consumed in high amounts, they form a considerable amount of flavonoid intake because of their high quercetin content. In contrast, tea and wine contain lower amounts of quercetin but in some countries, they are consumed at rather high quantities (Erlund 2004).

The average daily flavonol intake was estimated to be 15 – 30 mg/day for Denmark, and similar amounts for Holland and Japan. Finland had the lowest intake of 4 mg/d. However cultural diets often dictate which foods are consumed and so which flavonoids are most ingested. Japan may have had similar flavonol intake as European countries, but soy and soy foods are frequently consumed in Japanese diets and consequently they have the highest total flavonoid intake (63 mg/d), with isoflavones being the dominant flavonoid in their diet (Hertog et al. 1993b, Hertog et al. 1995, Beecher 2003). Cultural habits may remain a strong factor even after migration to a new country as East Asians in the US still have a relatively modest intake of

isoflavones compared with residents of Japan and non-Asian residents of the US (Beecher 2003).

In the United States, the estimated daily intake of flavonols is 20 - 22 mg, of which approximately 75% is quercetin (Sampson et al. 2002). Although this is in line with the Dutch diet, earlier estimations had been closer to a daily intake of 1 g/d in the US (Kuhnau 1976). This has since been stated as an over-estimation, most likely due to a lack of reliable analytical methods available at the time. But considering the vast number of flavonoids that exist in the diet, and that most studies base intake on only a very few select flavonoids, actual flavonoid intake is likely to be higher and perhaps levels close to 1 g/d are not completely unreasonable.

Food processing can affect quercetin concentrations. For example boiling foods like tomatoes or broccoli greatly reduces the flavonol content. This could be due to heat degrading the chemical structure or the polarity of quercetin causing it to leach into water. Onions, however, contain quercetin conjugates that lend it stability up to temperatures of 100 ^{o}C. Skins of fruits and leaves of vegetables are where quercetin is usually most concentrated so peeling or removing these will cause obvious losses in quercetin (Aherne and O'Brien 2002).

Daily polyphenol intake cannot be accurately assessed yet. However, with more extensive food composition databases being developed by each county, the precision of consumption data for various populations is also surely to improve.

1.6.2. Bioavailability

Plasma concentrations of quercetin tend to be in the nanomolar range with 50 - 80 nM being the average detected in overnight fasting humans volunteers (Erlund et al. 2002). However the baseline readings have the potential to increase into micromolar ranges with plasma levels reaching 1.5 μM after 28 days supplementation of a high quercetin dose (1 g/d) (Conquer et al. 1998) and 0.63 μM in another study where supplementation equivalent of 80 mg/d quercetin was given for 1 week (Kawabata et al. 2005). Reviews of bioavailability studies have reported that elimination of quercetin and its metabolites are relatively slow, with half lives of 11 – 28 hours which suggests it is possible for plasma accumulation to occur with repeated intakes (Manach et al. 2005).

Differences in individual gut physiology, including genetics (activity of transporters/enzymes) and microflora compositions can affect quercetin metabolism and absorption. This makes it difficult to predict bioavailability.

The hydrophobic nature of flavonoid aglycones mean that they can be transported directly across cell membrane of enterocytes by passive diffusion but studies have shown that the extent of absorption depends on the source of quercetin. Human ileostomy volunteers supplemented with various forms of quercetin showed absorption (oral intake - ileostomy excretion) of quercetin aglycone was approximately 52% from fried onions (contain quercetin-4'-glucoside and quercetin-3,4'-diglucoside), 24% from pure quercetin aglycone and 17% from rutin (quercetin-3-O-rutinoside) (Hollman et al. 1995). Furthermore, in another study, it was demonstrated that the sugar moiety itself has a major role in determining absorption (Hollman et al. 1999). The bioavailability of

quercetin-rutinoside was only 20% of that of quercetin-glucoside suggesting that conjugation to a simple monosaccharide like glucose allows the molecule to be absorbed in the small intestine whereas conjugation to a more complex sugar like the disaccharide rutinose requires further metabolism in the colon before it can be absorbed. Cell culture work implicated the sodium-glucose transporter-1 (SGLT-1) to be involved in the uptake of quercetin-glucosides. Using fluorescent microscopy and HPLC analysis, intestinal cell line Caco-2 and SGLT1 stable transfected chinese hamster ovary cells (G6D3) showed that quercetin-4'-beta-glucoside was actively transported by SGLT1 across the apical membrane of enterocytes (Walgren et al. 2000). This was corroborated in rat jejunal tissue using quercetin-3-glucoside which demonstrated a clear role of SGLT-1 for its mucosal uptake (Wolffram et al. 2002). Other transporters have also been implicated including glucose transporter 2 (GLUT2) (Wolffram et al. 2002) and multidrug resistance protein 2 (MRP2) (Walgren et al. 2000).

Quercetin glycosides that have not been absorbed by the small intestine pass into the large intestine where they are hydrolysed by colonic microflora liberating the aglycone. This has been suggested to take place in the lower ileum and the cecum which contain bacteria that produce β-glucosidases, α-rhamnosidases, and β-galactosidases enzymes capable of degrading flavonoid-glycosides (Bokkenheuser et al. 1987). Thereafter, the aglycone and its degradation products can be absorbed into the blood stream and transported to the liver by the portal vein or excreted in faeces. The liver is the main organ for flavonoid metabolism and it is here that quercetin is methylated and/or conjugated with glucuronic acid or sulphate. After hepatic processing quercetin conjugates can be excreted in urine or bile. Those in bile are metabolised by intestinal bacteria, hydrolysing glucuronic or sulphate conjugates

(Hackett 1986). The resultant metabolites can be re-absorbed and enter the entero-hepatic cycle. Thus the half-life of elimination can be affected by the source and type of quercetin presented (aglycone/glucose/glycose) and plasma levels have detected quercetin up to 48 hours after its consumption (Hollman et al. 1996). This again suggests that repeated intakes of quercetin glycosides, which take longer to process, can cause a build-up of plasma concentration.

1.6.3. Antioxidant properties

A full discussion of the biological activity of polyphenols is beyond the scope of this thesis, therefore this section will focus on the antioxidant mechanisms of polyphenols.

Historically, the biological effects of flavonoids have been controversial. With their discovery in the 1930/40s they were thought to have vitamin-like properties. Later, in the 1970s they were suspected mutagens and carcinogens, whereas the 1980s focused on their anti-mutagenic and anti-carcinogenic effects. More recent years have reported of their antioxidant capacities.

Renewed interest has stemmed from epidemiological studies showing an inverse relationship between increased consumption of flavonoids and reduced risk of cardiovascular disease. Quercetin content measured in various foods (and other flavonoids) by Hertog et al (1992) is generally used as a reference database. Assessment of flavonoid intake from dietary food can then be linked with health during a follow up period. The Zutphen Elderly Study is a published epidemiological study which assessed flavonoid intake of 805 men aged 65 to 84 years and found a

significant inverse relationship between dietary flavonoid intake and mortality from coronary heart disease (CHD) and a slightly weaker inverse association with incidence of myocardial infarction. The findings remained significant after correcting for known major confounding factors (Hertog et al. 1993a). In southern France, a high intake of saturated fatty acids was not correlated with increased CHD risk and a high consumption of red wine, rich in flavonoids, was proposed to explain this 'paradox' (Ferrieres 2004). Vascular endothelium is important for maintaining cardiovascular health and several clinical trials have examined the effect of flavonoid-rich food and drinks on endothelium-dependent vasodilation. One trial involving 66 patients with coronary heart disease, found that daily consumption of 4/5 cups (approximately 900 - 1250 ml) of black tea for four weeks significantly improved endothelium-dependent vasodilation compared to caffeine or hot water alone (Duffy et al. 2001). A smaller trial of 15 patients showed improvement of vasodilation and reduced LDL susceptibility to oxidation, after daily consumption of purple grape juice (3 cups ~ 640 ml) for 2 weeks (Stein et al. 1999).

Oxidation of low density lipoproteins (LDL) is a free radical process known as lipid peroxidation, which oxidises unsaturated fatty acids in LDL. This can initiate and develop atherosclerotic plaques, leading to cardiovascular diseases. Flavonoids exert their antioxidant activity here by interfering with this oxidation process, namely by rapidly donating hydrogen to radicals.

(i) R• + Flavonoid-OH → Flavonoid(O•) + RH

(ii) R• + Flavonoid(O•) → R-Flavonoid

R• denotes a free radical and O• denotes a reactive oxygen species

In reaction (i) the flavonoid radical intermediates are a more stable product therefore a new chain reaction cannot be easily initiated. The flavonoid radical intermediates themselves can also terminate further propagation by reacting with other free radicals (reaction ii).

Quercetin also reduced the cytotoxicity of oxidized LDL on lymphoid cell lines (Negre-Salvayre and Salvayre 1992). The exact mechanisms are not known but it is speculated that quercetin may block the generation of intracellular cytotoxic signals, possibly by inhibiting enzymes that are involved in signal transduction. An emerging view is that quercetin can affect protein kinases to modulate cell signalling. Signal transduction requires kinases to phosphorylate target proteins at specific sites to ultimately affect gene expressions. Recent *in vitro* work using primary cortical neuron has shown that quercetin and its metabolites can act on numerous kinases (phosphoinositide 3-kinase (PI3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC)) (Spencer et al. 2003, Williams et al. 2004) which may have profound effects on cellular functioning. Therefore it is clear that a better understanding is needed of quercetin as a cell-signalling modulator in its evaluation as a cardio-protectant.

The antioxidant efficiency of flavonoids depends on their chemical structure. Antioxidant structural elements include: a hydroxyl group in carbon position three, a C_2=C_3 double bond, a carbonyl group in carbon position four and poly-hydroxylation of the A and B aromatic rings (Rice-Evans et al. 1996, Cook and Samman 1996). Quercetin possesses all these characteristics making it one of the most potent natural antioxidants (Bravo 1998).

An important antioxidant mechanism of quercetin is as an effective iron chelator. Quercetin is suggested to interfere with iron absorption by forming insoluble iron complexes in the gastrointestinal lumen thus reducing iron bioavailability (Ma et al. 2010). This may have therapeutic implications in pathological conditions of iron overload. In cultures of iron-loaded rat hepatocytes, quercetin was efficient at sequestering iron from cells, revealing a good relationship between iron-chelation and cytoprotective effects (Morel et al. 1993). In the native state, iron can initiate free radical production by the Fenton and Haber-Weiss reactions but flavonoids like quercetin that possess both a C-4 carbonyl group and a C-3 or C-5 hydroxyl group, can chelate iron thus preventing iron-induced free radical formation (Afanas'ev et al. 1989).

Quercetin can protect against reactive oxygen species of different origins. Smoking is an environmental cue that generates free radicals in the body, damaging erythrocyte membranes as one of its many detrimental effects. Begum and Terao (2002) found that quercetin and its potential metabolites (quercetin-3-O-β-glucuronide and quercetin-3-O- β -glucoside) suppressed erythrocyte damage as well as the associated

membrane lipid peroxidation (Begum and Terao 2002). This shows that it is not only quercetin aglycone but also its conjugate metabolites that are capable of scavenging free radicals.

The abundance of flavonoids in the diet suggests they have been conserved to provide great nutritional benefits. Quercetin being the major constituent of flavonoid intake could be key in protecting against several chronic diseases. It has all the structural requirements to be a powerful antioxidant, and its cytoprotective effects have been noted in epidemiological and *in* vitro studies. However, further work is needed to better understand the effects of quercetin within the human body including rate and extent of its absorption, metabolism, and bioavailability.

Quercetin is able to suppress propagation of free radical formation by interfering with the lipid peroxidation process and chelating iron which may otherwise cause free radical production by the Fenton reaction. These provide some mechanisms for the beneficial link of flavonoids with reduced heart diseases observed in epidemiological studies.

1.7. Polyphenol and iron interaction

1.7.1. Nuclear factor erythroid 2-related factor 2 (Nrf2)

The nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that functions as a key inducer of antioxidant and detoxifying enzymes through its binding to antioxidant response elements (ARE). During conditions of oxidative stress, the Nrf2 signalling pathway is activated (Figure 1.14). In the cytoplasm, kelch-like ECH-associated protein 1 (Keap1) interacts with Nrf2 as an inactive complex, promoting rapid Nrf2 ubiquitination and degradation (Itoh et al. 1999). Increased intracellular ROS weakens this Keap1-Nrf2 interaction and Nrf2 is stabilised, allowing it to dissociate from Keap-1 and translocate to the nucleus. Here it associates with its transcriptional partner, Maf proteins, forming a heterodimer that binds to antioxidant response elements of DNA, activating transcription of a whole host of cytoprotective genes (Itoh et al. 1997).

Recently it has been suggested that quercetin may be able to activate this Nrf2 pathway (Granado-Serrano et al. 2012). In the human hepatoma cell line (HepG2) it was shown that 10 μM quercetin was able to regulate Nrf2 by up-regulating the phosphorylation and translocation of Nrf2. This phosphorylation process has been described as critical for its nuclear translocation and transcriptional activity (Pi et al. 2007). Therefore it seems as though the actions caused by quercetin on Nrf2 may be important for defending cells against ROS insult and maintaining a favourable redox balance.

Ferroportin is essential for the control of iron efflux therefore its possible regulation by Nrf2 is of major interest. Bioinformatics analysis revealed four putative MARE/ARE (Maf Recognition Element/Antioxidant Responsive Elements) motifs, upstream of the macrophage FPN transcription site, one of which is located within a highly conserved FPN promoter region (Marro et al. 2010). Using luciferase constructs, mutation of this MARE/ARE sequence (at position -7007/-7016) resulted in abolished haemoglobin-induced activation of luciferase activity suggesting it is essential for regulating FPN transcription in response to haemoglobin. Further studies are required to see if non-haem iron is able to cause similar activation and whether quercetin influences Nrf2-induced FPN regulation.

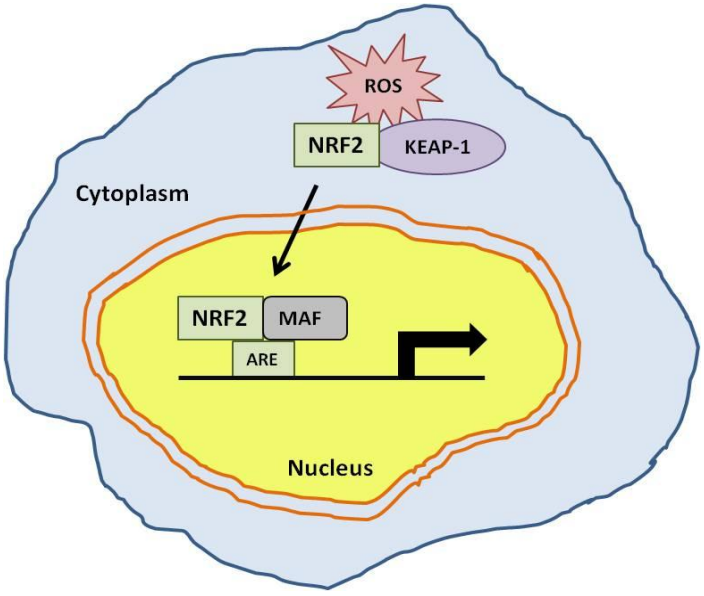


Figure 1.14 Activation and induction of Nrf2 signalling pathway

Nrf2 is kept in an inactivate complex with Keap-1 in the cytoplasm. ROS stimulates dissociation of Nrf2 and translocation to nucleus. Nrf2 binds with Maf protein, and binds to ARE sequences in the promoter region of genes encoding antioxidant and detoxifying enzymes.

1.7.2. Polyphenol and iron interaction in the diet

Polyphenols exert a number of effects, and as previously mentioned, polyphenols can chelate iron, which is one way they affect non-haem iron bioavailability. Another mechanism more recently shown is that quercetin may act as a substrate for DcytB, the endogenous apical ferric-reductase (Vlachodimitropoulou et al. 2010). This may increase the reducing potential of DcytB, providing more Fe^{2+} for DMT1-mediated cellular uptake. Quercetin being lipophilic can easily cross lipid bilayers but it has been stipulated that when it binds to iron, it forms stable quercetin-iron complexes which are redox-inactive (Baccan et al. 2012). These complexes may be able to enter cells via glucose transporter proteins (GLUTs). Quercetin can utilise GLUT-1, -3 and -4 to enter cells and acts as an inhibitor of dehydroascorbate and glucose transport when using the same transporters (Strobel et al. 2005, Vlachodimitropoulou et al. 2011). GLUT-1, which is the only endogenously expressed glucose transporter in MDCK cells (Madin Darby canine kidney cells), has been shown to be capable of transporting the chelated-complex in either direction across the cell membrane (Vlachodimitropoulou et al. 2011). The possibilities then are that these complexes are too large to be exported out by ferroportin leading to increased intracellular iron levels, or that these complexes may be exported out by GLUT-1 thus quercetin acts as a siderophore (a compound that bind iron for removal or delivery to cells). Another study found that glycosylated quercetin such as rutin are able to pass cell membranes but only free quercetin is able to access the cytosol to decrease intracellular labile iron pools (Baccan et al. 2012).

Quercetin interaction with iron has shown itself to be multi-faceted. Quercetin can directly scavenge free radicals; it is an iron chelator and can thereby inhibit iron-induced free radical production; it is an electron donor to DcytB which in turn reduces iron to a more soluble form (Fe^{2+}). Quercetin is membrane-permeable via GLUTs, so can easily enter the cell cytosol for iron chelation. Quercetin-iron complexes are stable, non-reactive and can possibly enter/exit cells by GLUT-1. Aglycone quercetin can decrease iron present in the cytosol by speeding up its passage across the cell membrane and loading on to the iron transport protein transferrin.

Although there are several mechanisms in place to regulate iron, imbalances can still occur. Iron overload occurs in primary hereditary haemochromatosis, which is caused by mutations in genes important for iron regulation. Mutations include genes encoding for haemojuvelin, hepcidin, transferrin receptor-2 (Tfr2) and ferroportin. Lack of hepcidin (or low levels) means there is no negative signal to stop iron absorption even when the systemic level of iron is high. Mutations in ferroportin can impair iron export, giving rise to intracellular iron accumulation, or cause ferroportin resistance to hepcidin, which results in constant iron export, iron-loading the rest of the body. Secondary iron overload can be caused by repeated blood transfusions in haematological disorders like thalassaemia (Papanikolaou et al. 2005). Current treatment for secondary iron overload includes chelators such as desferrioxamine (DFO), deferiprone or deferasirox. DFO has to be administered intravenously or subcutaneously which has poor patient compliance. Deferiprone has been an oral alternative but severe gastrointestinal complaints are frequent with commonly reported side effects of nausea and vomiting (Eshghi 2007). This, alongside patient

neglect to take the drug three times a day for prolonged periods causes problems with prescribed deferiprone regimen.

There are certain conditions where iron overload may occur in select areas without causing general hyperferraemia. In anaemia of chronic disease, plasma iron levels are very low but macrophages contain high amounts by withholding iron. Certain neurodegeneration disorders accumulate iron within the brain. Using general iron chelators for these disorders may be inappropriate; rather a better therapeutic approach would be to target its redistribution (Kakhlon et al. 2010). Ideally, to be able to do this, chelators should be able to penetrate iron-overloaded tissue, form stable and redox-inactive species (to decrease intracellular labile iron) and transfer it to circulating transferring (Baccan et al. 2012) . Quercetin meets all the requirements and its role as a ‘shuttle’ for iron may be of particular use for iron-redistribution therapy.

Polyphenols constitute one of the most ubiquitous compounds in the plant kingdom thus forming an integral part of human diets. Quercetin, belonging to the flavonoid group, represents one of the most common and widely distributed plant polyphenols. It can bind iron by forming complexes through its carboxyl and hydroxyl groups, and consequently is capable of interfering with intestinal iron absorption. Polyphenol supplementation is becoming more frequent in food and health industries therefore the nutritional significance of quercetin and its potential health benefits requires more detailed studying. As deregulation of iron is implicated in the development of many diseases, polyphenols may have important applications in their treatment. To fully understand the actual significance of polyphenols, it is necessary to investigate their

mechanisms of action. It is important to define how dietary polyphenols interact with our intake of essential nutrients like iron as only then can we correctly recommend and optimise intake for different health and disease states. These mechanistic factors comprise the main body of research for this thesis.

1.8. Aims of study

The aim of this study was to use biologically relevant *in vitro* models to elucidate the mechanisms underlying the effects of quercetin, the most abundant flavonoid, on iron metabolism.

The Caco-2 cell line was utilised as a model of intestinal enterocytes to investigate the effects of quercetin on iron bioavailability and gene expression. To deduce the molecular mechanisms of quercetin on iron absorption, cells were transfected with ferroportin promoter constructs. Ferroportin 3'UTR and activation of miRNAs were also explored.

HepG2 hepatocarcinoma cells were used to model hepatic iron storage. As the site of hepcidin production, effects of quercetin on hepcidin expression and intermediates in the hepcidin signaling cascade were investigated. Liver pathology is often changed in iron-overload diseases thus the effect of quercetin on iron-induced changes in cell proliferation and viability were also examined.

Thp1 cell line represented macrophage iron-recycling system and was used to study changes in iron transporter gene expression induced by iron and quercetin and their effects on FPN promoter.

Hypothesis:

The starting hypothesis was that quercetin would alter the expression of genes involved in iron metabolism via its action as an iron chelator, thereby reducing intracellular iron levels.

An alternative hypothesis is that quercetin acts independently of changes in cellular iron status and exerts direct effects of gene expression.

2. Materials and methods

2.1. Cell Culture

2.1.1. Cell culture material

Table 2.1. Cell culture material and reagents

Material	Description	Supplier	Cat
Media	Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen	41965-062
Media	Roswell Park Memorial Institute medium (RPMI)	Invitrogen	21875-034
Serum	Fetal Bovine Serum (FBS), Heat-inactivated	Invitrogen	10108-165
Trypsin	TrypLE(TM) Express	Invitrogen	12605-010
Supplement	Penicillin 10,000 U/ml (100x)	Invitrogen	15140-122
Supplement	Streptomycin 10,000 µg/ml (100x)	Invitrogen	15140-122
Supplement	L-Glutamine 200 mM (100x)	Invitrogen	25030-024
Supplement	MEM Non Essential Amino Acids 10 mM (100x)	Invitrogen	11140-035
Supplement	Plasmocin, prophylaxis 2.5mg/ml	InvivoGen	Ant-mpp
Dye	Trypan blue	Sigma-Aldrich	T8154
PMA	phorbol 12-myristate 13-acetate	Sigma-Aldrich	P1585
DMSO	Dimethylsulfoxide	Sigma-Aldrich	D8418
Treatment	Quercetin	Sigma-Aldrich	Q4951-10G
Treatment	Hemin	Sigma-Aldrich	H9039-1G
Treatment	Hepcidin	Peptide international	PLP-4392-s
Treatment	LY294002 (PI3K inhibitor)	Calbiochem	440204
Treatment	HIF-2α inhibitor	Calbiochem	400088
Treatment	HIF-2α inhibitor	Calbiochem	400087

2.1.2. Cell culture

All cells were maintained at 37ºC in an atmosphere of 95% air and 5% CO2 using Nunc tissue culture plastics. The media (DMEM) contained 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine (10% v/v), 100 U/ml penicillin (10% v/v), 100 µg/ml streptomycin (10% v/v), and 2.5 mg plasmocin (0.2% v/v). Media was refreshed every other day. All supplements were filtered through a 0.2 µm pore size filter before adding to media. Medium was changed to serum-free 24 hours before treatment to cause growth arrest and therefore keep all the cells in the same phase of the growth cycle. To subculture Caco-2 and HepG2, media was removed and briefly washed with PBS for 5 seconds. 0.2% (v/v) Trypsin was added to stimulate detachment for a period of 10 min, after which an equal volume of serum-containing media was added to deactivate trypsin activity. Cells were collected in a 50 ml falcon tube and centrifuged at 900-1000 rpm for 5 min. Supernatant was removed and cell pellet was resuspended in 10 ml of new culture medium and cell numbers were determined with a haemocytometer.

A Trypan blue exclusion test was used to estimate cell number and viability. Viable cells have an intact cell membrane and appear clear under the microscope whereas dead cells have a damaged membrane, allowing the Trypan blue to leak in and stain the cell. 10 µl of cell suspension with Trypan blue in a 1:1 ratio at 0.2% (w/v) was loaded onto a haemocytometer. The haemocytometer contain $1x10^{-4}/cm^3$ of cell suspension and counting only viable cells, the number of cells in the original sample was calculated as follows: Cells per ml = (Number of live cells counted) x 10^4 X (dilution factor). The dilution factor was always 2.

Thp1 remained in cell suspension. To renew media every 2 days, cells were collected in a 50 ml falcon tube, centrifuged, supernatant removed, fresh media added and then placed back in flask. The same occurred for sub-culturing, except cells underwent Trypan blue test and haemocytometer counting to determine quantity. This occurred every 3-4 days.

Stocks of all cells were stored at a density of 1 x 10^7 cells/ml of freezing medium (30% FBS supplemented media and 5% DMSO) in cryovials. These were initially stored at -80 °C in a "Mr. Frosty" 5100 Cryo 1°C Freezing Container (Nalgene) for 24 hours and then transferred to liquid nitrogen for long term storage.

2.1.3. Cell culture for experiments

For RNA and protein isolation, Caco-2 cells were seeded for 21 days in normal culture medium to allow full differentiation (density stated in Table 2.1.2). Media was changed to serum-free on day 21 to arrest cell growth and treatment with normal media occurred on day 22. HepG2 cells were plated for two days followed byserum-free media for one day and thereafter treatment with normal media. THP1 cells were seeded for 24 hours, 100 nM PMA in DMSO was added for a further 24 hours to promote differentiation of monocytes into macrophages. The PMA-containing media was removed and replaced with serum-free media for 24 hours before treatment.

Table 2.2 Cell plating density (cells/well) and time required for growth

Cell type	6-well plate	12-well plate	Growth period
Caco2	4 X 10^4	2 x 10^4	21 days
HepG2	5 X 10^5	2.5 X 10^5	2 days
THP1	1 X 10^6	5 X 10^5	1 day (+1day PMA)

Cells were seeded at different densities according to the plate size and cell type

2.2. q-PCR (Real time PCR)

2.2.1. RNA isolation

TRI Reagent (Sigma) was used to isolate RNA from cells. 1 ml of TRI Reagent was added to each well of a 6-well plate (0.5 ml for 12-well plate). Cells were incubated at room temperature for 5 min to allow complete dissociation. The solution was transferred into a 1.5 ml Eppendorf and 200 µl/ml of chloroform was added (1:5) and thoroughly mixed by hand for 15 seconds to precipitate proteins. After 10 min room temperature incubation, samples were centrifuged at 12000 *g* for 15 min at 4°C causing the solution to separate into 3 layers. The colourless upper aqueous phase containing the RNA was transferred to a fresh Eppendorf. RNA was precipitated by adding 500 µl of ice-cold isopropanol (Sigma, Cat No. 19516) to the Eppendorf at room temperature for 10 - 15 min and then centrifuging at 12000 *g* for 10 min at 4°C. Isopropanol was removed and the pellet washed with 1 ml of 75% ethanol (Sigma-Aldrich, Cat No. E7023) by vortexing followed by centrifugation at 7500 *g* for 5 min at 4ºC. Ethanol was discarded and the final RNA pellet air-dried. To dissolve RNA, 20 - 30µl RNAse-free water was added and kept at 60ºC for 5 - 10 min. After this, RNA samples were kept on ice at all times.

Concentration of extracted RNA was measured using a NanoDrop® spectrophotometer ND-1000 (NanoDrop, USA). Quality was assessed by using the A260/280 and A230/260 ratio. RNA with a ratio higher than 1.9 for A260/280 confirms there is no protein contamination and a ratio higher than 1.5 for A230/260 indicates no solvent contamination.

2.2.2. Reverse transcription

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA by using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Cat No. 4374966). Using the Nanodrop RNA measurements, the volume needed for 1 µg RNA was calculated and transferred to a PCR tube and made up to 10 µl by adding RNAse-free water. Following the manufacturer's protocol, a master mix was made (Table 2.3) and 10 µl of this was added to each tube PCR tube. Tubes were centrifuged and placed in a thermal cycler in the following optimised conditions: Primer incubation; 25ºC for 10 min, Reverse transcription; 37ºC for 120 min, and enzyme inactivation; 85ºC for 5 min. Samples were kept at 4ºC for immediate use or at -20 ºC to be used later.

Table 2.3 Reverse transcription 2x master mix components

Component	Volume (µl)/reaction
10×RT Buffer	2.0
25× dNTP Mix (100mM)	0.8
10×RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H2O	3.2
Total per reaction	10.0

2.2.3. Real-time PCR

The fluorescence readings were recorded at the annealing step (Table 2.5). Data was analysed using the ABI Prism software. The gene expression data were normalized to individual 18S RNA expression.

Real time PCR was performed on 1µl of cDNA reaction mixture using the ABI Prism 7000 sequence detection system thermo cycler (Applied Biosystems) coupled with SYBR green technology (Qiagen GmBH, Germany). SYBR green is a fluorescent dye that binds to the minor groove of DNA and fluoresces only when bound to double stranded DNA. The intensity of the signal is proportional to the amount of amplified DNA detectable during each cycle and the cycle threshold is defined as the first cycle in which there is a significant increase of the signal. Passive-reference dye called ROX normalises for any non-PCR fluorescence, for example variations in volumes between wells. By plotting Ct values against a standard curve, it is possible to calculate the relative quantity of a specific gene.

Samples were prepared in 96-well PCR semi-skirted plates (Starlab UK) with a final volume of 25 µl. Master mixes were prepared for each primer pair as described in Table 2.2.2. cDNA was directly used for each gene but was diluted for housekeeping gene 18S ribosomal RNA (1:100 dilution) due to its extremely high expression. A standard curve was generated for each gene using a set of five standard dilutions (1, 0.1, 0.01, 0.001, 0.0001) which were prepared from a pool of templates. This was run on each plate alongside the unknown samples to quantify efficiency of amplification as well as two non-template controls which did not contain any sequence to check for contamination.

Table 2.4 Components of qPCR reaction plate

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix	12.5 µl	1x
Forward Primer	1.25 µl	0.5 µM
Reverse Primer	1.25 µl	0.5 µM
RNase-free water	10 µl	----
Template (cDNA or Standard)	1 µl	~1000 ng/reaction
Total volume	25 µl	----

qPCR plates were centrifuged and placed in the ABI Prism 7000 real-time cycler with the following programme:

Table 2.5 q-PCR cycling parameters

Cycles	Temp (°C)	Time	Comment
1	95	2 min	Initial activation
40	94 60 72	15 sec 30 sec 30 sec	Denature Annealing Extension
1	95 60 95	15 sec 20 sec 15 sec	Dissociation

Data was collected for each cycle at the end of extension step (72°C for 30 sec).

Table 2.6 Primer sequences used in this study

Primer	Forward (5' →3')	Reverse (5' →3')
18S	AACTTTCGATGGTAGTCGCCG	CCTTGGATGTGGTAGCCGTTT
BMP6	CCGTGTAGTATGGGCCTCAGA	TCACAACCCACAGATTGCTAGT
DcytB	GCATCGCCATCATCGTCTACA	ACGGCCACCACAGAGATAATT
DMT1+IRE	AGTGGTTTATGTCCGGGACC	TTTAACGTAGCCACGGGTGG
Ferroportin (FPN)	CCACAATACGAAGGATTGACCA	GGA CGT ACT CCA CGC ACA TG
FPN1A	AAAGAAGACCCCGTGACAGC	TCCCCTTGTTTGTTCTGATG
FPN1B	GCCGGTTGGAGTTTCAATGT	TCCCCTTGTTTGTTCTGATG
Hephaestin	CACACCATGCACTTTCATGC	GCATGCACATGGTCAGTCAC
Hepcidin	CTGCAACCCCAGGACAGAG	GGAATAAATAAGGAAGGGAGGGG
SMAD4	GATACGTGGACCCTTCTGGA	ACCTTTGCCTATGTGCAACC

2.3. Western blotting

2.3.1. Protein isolation

After treatment, media was removed from 6-well plates and washed briefly with PBS. 100 µl of protein lysis buffer (Table 2.7) was added per well and placed on a rocker for 10 min. A cell scraper was used to detach the cells, and collected into a corresponding Eppendorf where it was homogenised through a 25 gauge needle to promote cell lysis. After centrifugation (13,000 rpm for 10 min at 4°C) to remove cell debris, the supernatant was transferred to a fresh Eppendorf and kept on ice for quantification.

Table 2.7. Protein lysis buffer

Material	Description	Conc.	Supplier	Cat No.
PBS	Phosphate buffered saline	N/A	Oxoid	BR0014G
PI	Protease Inhibitor Cocktail	1:100	Sigma-Aldrich	P8340
SDS	Sodium dodecyl sulphate	1:1000	Sigma-Aldrich	71727

2.3.2. Nuclear and cytoplasm protein extraction

Nuclear proteins are more concentrated in nuclear extracts than in whole cell lysates. To isolate nuclear and cytoplasmic proteins, NE-PER Nuclear and cytoplasmic Extraction Reagent (thermo Scientific Cat No 78833) kit was used and the manufacturer's protocol followed.

Caco-2 cells were harvested with trypsin-EDTA and centrifuged at 500 x g at 4°C for 5 min. All samples and extracts from this point forward were kept on ice and all centrifugation performed at 4°C. The cell pellet was suspended in PBS and centrifuged at 500 x g for 2 - 3 min. The supernatant was carefully discarded to leave the pellet as dry as possible. Protease inhibitor was added to CER I and NER just before use. 100 µl ice cold CER I was added to the pellet and vortexed to fully suspend the pellet and kept on ice for 10 min. Ice cold 5.5 µl CER II was added to the sample, briefly vortexed for 5 seconds before incubating on ice for 1 minute. It was briefly vortexed again, and centrifuged at the highest speed for 5 min. The supernatant (cytoplasmic extract) was immediately transferred to a fresh Eppendorf and kept at 4°C until use.

The remaining cell pellet was suspended in 50 ul ice-cold NER and vortexed. The sample was kept on ice and vortexed for 15 seconds, every 10 min, for 40 min. After this, the sample was centrifuged at the highest setting for 10 min and the supernatant (nuclear extract) immediately transferred to a fresh Eppendorf. Different volumes of reagents could be used depending on cell volume as long as a volume ratio of CER I:CER II:NER at 200:11:100 µl was maintained. Extracts were stored at -80°C until use.

2.3.3. Protein quantification

Protein concentration was determined using the BioRad Protein Assay Kit (Bio-Rad, Germany, Cat No 500-0006), based on the Coomassie blue binding method which causes a shift in absorbance of the Coomassie reagent from red to blue when it binds to protein (Bradford, 1976) at OD_{595}. A 1 in 5 dilution of the Bio-Rad dye was made (provided as a 5 x concentrated solution) with ddH_2O to be used for the assay. 10 µl of protein sample were dissolved in 90 µl of 200 mM Sodium Hydroxide (NaOH). Serial dilutions of bovine serum albumin (BSA) were used as standard concentrations (stock was 1 mg/ml; dilution range: 0, 5, 10, 20, 50, and 75 µg) and made up with NaOH to a final volume of 100 µl. 4 ml of diluted Bio-Rad solution was added to each tube. The absorbance was read at 595 nm and plotted against the standard concentrations. This generated a standard curve to quantify protein concentration of the samples.

2.3.4. SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is formed by the polymerization of acrylamide and bis-acrylamide, to separate proteins

according to their size. Normally a 10% gel was used unless the protein had a molecular weight greater than 120 kDa in which case an 8% gel was used. Table 2.8 shows the materials used to prepare the gel.

Table 2.8 Materials and volumes required to make 1 SDS-PAGE resolving gel

Material	Description	Supplier	Cat No.	Volume for 1 gel	
				8%	10%
Distilled water	---	---	---	4.095 ml	3.525 ml
Acrylamide 40%	Acrylamide 40%, Acrylamide/Bis Solution, 29:1	Bio-Rad	161-0146	1.575 ml	1.875 ml
Tris 1.5M pH8.8	Tris Base (1.5 M, pH= 8.8)	VWR	103156X	2.047 ml	1.95 ml
SDS 10%	Sodium dodecyl sulphate, 10% solution	Sigma-Aldrich	71727	78.75 µl	75 µl
APS 10%	Ammonium Persulphate	Sigma-Aldrich	A3678	78.75 µl	75 µl
TEMED	N,N,N',N' tetramethylethylenediamine	Sigma	T9281	10.5 µl	10 µl

A gel casting cassette was assembled using 2 glass plates with a silicone rubber seal in-between and clipped together. The resolving mixture (Table 2.8) was poured in between the glass plates and a 14-well comb was inserted at the top. The solution was left to set for 15 - 30 min at room temperature after which the comb and seal were removed and any gel residues washed away with water. It was then placed in an electrophoresis gel tank. 1 x running buffer (Table 2.10) was poured into the tank till the wells were completely immersed.

A minimum of 20 µg of protein was mixed with an equal volume of 2 x Laemmli loading buffer (Table 2.9) and loaded on to the gel along with 7 µl of rainbow molecular marker (GE Healthcare, USA, Cat No. RPN800E) in the first well of each gel. The gels were run at 30mA (per gel), 220 V for 90 min or until the bromophenol blue dye in the Laemmli loading buffer reached the bottom of the gel.

Table 2.9 2x Laemmli loading buffer

Material	Final Conc	Supplier	Cat No.
SDS (w/v)	2%	Sigma-Aldrich	71727
Tris (pH=6.8)	62.5 mM	VWR	103156X
Glycerol (v/v)	25%	Sigma-Aldrich	G5516
Bromophenol blue (w/v)	0.01%	VWR	20017
β-mercaptoethanol (v/v)	14.4 mM (10%)	Sigma-Aldrich	M7522

Table 2.10 Running buffer

Material	Final Conc	Prep.	Supplier	Cat No
Tris Base	25 mM	3 g/l	VWR	103156X
Glycine	250 mM	19 g/l	Sigma-Aldrich	G8898
SDS	0.02% (w/v)	0.2 g/l	Sigma-Aldrich	71727

Table 2.11 Transfer buffer

Material	Final Conc	Supplier	Cat No
Tris Base	25 mM	VWR	103156X
Glycine	190 mM	Sigma-Aldrich	G8898
SDS	0.037% (w/v)	Sigma-Aldrich	71727
Methanol (v/v)	20%	VWR	101586

Table 2.12 Phosphate buffered saline-Tween20 (PBST)

Material	Final Conc	Supplier	Cat No
PBS (1x)	N/A	Oxoid	BR0014G
Tween 20 (v/v)	0.1%	Sigma-Aldrich	P5927

Proteins were transferred from the gel to nitrocellulose membrane (GE Health care, USA) using a Trans-Blot semi dry electrophoresis transfer cell. To do this, gels were allowed to equilibrate in transfer buffer (Table 2.11) for 15 min to remove any salt or detergent. Nitrocellulose membrane was cut to the size of the gel and 2 pieces of slightly larger filter paper were soaked in transfer buffer for 2 min. The pre-soaked filter paper was placed on the machine and the nitrocellulose on top of the filter paper. The gel was placed gently on top of the nitrocellulose membrane and another filter placed on top of the gel. Any air bubbles between the layers were rolled out using a pipette. The cathode lid was placed on top and the machine set to transfer at 70 mA/gel for 70 min.

2.3.5. Protein detection

Nitrocellulose membrane was incubated in 5% blocking buffer (2.5 g in 50 ml PBST) for a minimum of 1 hour at room temperature on a shaker. In a 50 ml falcon tube, primary antibody was diluted in 1% milk and the nitrocellulose membrane placed inside on a roller for overnight incubation at 40 rpm at 4 °C.

The membrane was washed three times for 5 min with PBST and then placed in a 50 ml tube with 1% milk containing the appropriate HRP-conjugated secondary antibody

at 40 rpm for one hour at room temperature. The membrane was finally washed 3 times with PBS and protein bands developed using the ECL plus western blotting reagent kit (GE Healthcare, USA, Cat No. RPN2132). Following the manufacturer's protocol, developing (A) and enhancer solutions (B) were mixed in a 40:1 ratio and added to the membrane for 5 min. Excess solution was drained and the nitrocellulose membrane was placed between 2 transparent plastic sheets inside a developing film cassette. In a dark-room, X-ray film (Hyperfilms ECL, GE Healthcare, USA, Cat No. 28906837) was exposed to the membrane to detect chemiluminescence. The incubation period varied for different proteins. For visualisation of the protein bands, the film was placed in developing solution (Kodak processing chemical, GBX developer, Sigma-Aldrich, USA, Cat No. P7042) till bands appeared, then washed with distilled water and fixed in fixing solution (Kodak processing chemical, GBX fixer, Sigma-Aldrich, USA, Cat No. P7167).

The relative movement of protein bands were compared against the molecular weight marker to ensure the correct protein was being analysed. Developed films were scanned and the intensity of bands was numerically quantified using Image J software. Data was normalised to a housekeeping protein.

Table 2.13 Antibodies used in western blotting system

Antibody	Type	Raised in	MW (kDa)	Dilution	Supplier	Cat No.
β-Actin	Primary	Rabbit	42	1: 1000	Sigma-Aldrich	A2066
B-tubulin	Primary	Rabbit	55	1:1000	Cell Signaling Technology	5346S
DMT1±IRE	Primary	Rabbit	65	1:500	Alpha-Diagnostics	NRAMP24-A
Hephaestin (Hp)	Primary	Rabbit	155	1:1000	Alpha-Diagnostics	HEPH11-A
Ferroportin	Primary	Rabbit	110	1:1000	Alpha-Diagnostics	MTP11-A
Nfr2	Primary	Rabbit	70 - 100	1:1000	Santa Cruz Biotechnology	sc-722
IRP2	Primary	Goat	105	1:500	Santa Cruz Biotechnology	sc-14221
Goat anti rabbit	Secondary	----	----	1:1000	Dako Ltd	P044801
Rabbit anti goat	Secondary	----	----	1:1000	Dako Ltd	P 0160

2.4. Ferritin assay

Following treatment, protein was isolated and quantified from Caco-2 cells as described in section 2.3.1. To measure changes in ferritin levels caused by these treatments, a ferritin assay was performed using the Ferritin Linked Enzyme Immunoabsorbent Assay (ELISA) Spectro-ferritin kit (Ramco Laboratories Inc, ATI Atlas Ltd UK, Cat No. S-22). The manufacturer's protocol was followed. Briefly, serial dilutions of human spleen ferritin were provided in the kit to create to a standard curve (6, 20, 60, 200, 600, and 2000 ng/ml). 10 µl of sample lysate was added to separate microwells provided in the kit; these were coated with anti-ferritin antibody. 200 µl of conjugated anti-human ferritin was added to all wells and the plate was incubated at 200 rpm on a shaker for 90 min at room temperature. The wells were washed 3 times with distilled water and excess liquid was drained on tissue paper. 200 µl of substrate solution was added to each well and the plate incubated for 30 min at room temperature at 200 rpm. The colour was developed by adding 100 µl of 0.24% potassium ferricyanide and mixing thoroughly. A well containing 200 µl substrate solution and 100 µl potassium ferricyanide was used as a blank. The plate was read within 1 hour using a HT-1 plate reader (Synergy, UK) at a wavelength of 500 nm and corrected to 600 nm. The results were calculated using the standard curve (absorbance of standards plotted against their ferritin concentrations) and normalised to protein concentration before statistical analysis.

2.5. ^{55}Fe uptake

Caco-2 cells were grown on Transwell inserts with 3.0 µm pore size (Costar, Fisher Scientific Ltd, Cat No TKT-526-020J) in 6-well plates. Following treatment with quercetin (0, 10 µM, and 100 µM) for 24 hours, cells were washed with HEPES-buffered salt solution (HBSS) pH 7.5, (containing NaCl 140 mM; KCl 5 mM; Na_2HPO_4 1 mM; $CaCl_2$ 1 mM; $MgCl_2$ 0.5 mM; HEPES 10 mM; D-glucose 5mM and bovine serum albumin (BSA) 0.2% w/v). To achieve a transepithelial gradient, 1.5 ml MES buffer (same composition as HBSS but HEPES is substituted for MES (2-(*N*-morpholino) ethanesulfonic acid) and no BSA) pH 6.5 was added to the apical chamber and 2.5 ml HBSS (pH 7.5) to the basolateral chamber and cells were pre-incubated at 37ºC for 20 min.

Iron uptake was initiated by adding to the apical media, a mixture of ferrous sulphate:ascorbate (10:100 µM) and 37kBq/ml radioactive $^{55}FeCl_3$ to provide approximately 500,000 DPM/well (disintegration per minute) ($FeSO_4$ - Sigma UK, Cat No F8048-500G; ascorbic acid - Sigma UK, Cat No A4034-500G; Iron-55 Radionuclide, 1mCi (37MBq), PerkinElmer, Cat No. NEZ043001MC).

To measure uptake, cells were incubated at 37ºC for 20 min and to measure transport, the incubation period was 120 min. Incubation was terminated by washing cell monolayers three times with ice-cold HBSS, cutting the Transwell membrane and placing membrane in to a corresponding well on a 6-well plate that contained 200 mM NaOH where they were kept overnight at 4ºC to allow cell lysis. Basolateral media

was also collected in corresponding Eppendorfs to determine iron efflux. The next day, membranes were thoroughly scraped and cell lysates passed through a 1 ml syringe to further lyse cells. For iron uptake (retention) 150 µl cell lysate was added to vials containing 5 ml of scintillant and for iron efflux 250 µl of basolateral media was added to vials of 5 ml scintillant. Iron transfer was calculated as the sum of the iron retention plus basal efflux. 15 µl of radioactive iron mixture ($^{55}FeCl_3$, $FeSO_4$-ascorbate) was used to determine starting activity. A LS100C Liqiud Scintillation Counter (Beckman-Coulter, UK) was used on the tritium channel in 5 ml of scintillant for 10 min per vial. Cell lysates were also used to measure protein concentration using the Bio-Rad method previously described and uptake and transport measurements were corrected to their protein levels.

2.6. MTS cell viability assay

Caco-2 cells were grown on 24-well plates and after 24 hour treatment, cell viability was assessed using an MTS assay. The CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega UK Ltd, Cat No G3580) uses the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] to provide a colourimetric method for determining cell viability. Tetrazolium salts, such as MTS, are pale yellow salts that can be reduced to form blue coloured formazan products by the cytochrome system in only live cells, thus the intensity of the colour produced is directly proportional to cell viability.

After treatment, 100µl of growth media was removed from each well and placed in a well on a clear 96-well plate. 15 µl of thawed MTS solution was then added to each well and the plate incubated in the darkat 37°C for 3 hours. The absorbance was read

at a wavelength of 485nm using a fluorescence plate reader (FLUOstar OPTIMA, BMG Labtech).

2.7. BrdU cell proliferation assay

The BrdU proliferation assay (Calbiochem, Cat No HTS01) is an immunoassay that incorporates bromodeoxyuridine, a thymidine analogue, into newly synthesised DNA strands. This allows us to assess the amount of cells that are actively synthesising DNA and therefore proliferating.

100 µl of HepG2 cells were seeded at 10^4 – 10^6 /well in 96-well plate and left for 24 hours for cell attachment. Stock BrdU label provided in the kit was diluted 1:2000 into fresh serum-free media to produce a working stock, 20 µl of which was pipetted into each well, alongside the treatment. Following 24 hour co-incubation, well contents were removed and the plate blotted on tissue paper. 200 µl of Fixative/Denaturing Solution was added to each well and left for 30 min at room temperature. Well contents were removed again. 100X Anti-BrdU Antibody was diluted 1:100 in Antibody Dilution Buffer, and 100 µl of this solution added to wells. The plate was incubated at room temperature for 1 hour. A working solution of 1X Wash Buffer was prepared by mixing 25 ml of 20X concentrated solution with 475 ml deionised water. After 1 hour incubation, wells were washed with the wash buffer three times and the plate blotted on paper towels. Reconstituted Peroxidase-linked Goat Anti-Mouse IgG was diluted 1:1000 in Conjugate Dilutent and passed through a 0.2 µm filter. 100 µl of this solution was added to wells and the plate incubated at room temperature for 30 min. Wells were washed three times with 1X Wash Buffer and the whole plate

flooded with deionised water. The plate was inverted over a sink to remove contents and then blotted on paper towels. A fluorogenic substrate working stock solution (9 parts fluorogenic substrate to 1 part fluorogenic peroxide solution) was prepared and 100 µl of the solution added to wells. The plate was incubated at room temperature for 30 min. 100 µl of Stop Solution was then pipetted into wells in the same order as the Fluorogenic Substrate working stock solution. The plate was measured using a fluorescence plate reader (BMG FLUOstar Fluorometer, OPTIMA, Labtech) measuring excitation at 355 nm and emission at 460 nm. Wells were read within 30 min of adding the Stop Solution for accurate readings.

2.8. Ferroportin promoter

Three plasmids were used in this study to assess the effect of quercetin and other treatments on ferroportin promoter: pGL4 empty plasmid as a control, pGL4-FPN1Bpm (FPN1B) and pGL4-FPN1Apm (FPN1A). FPN1B lacked the iron response element (IRE) whereas FPN1A contained the IRE. These were kindly gifted by Dr De-Liang Zhang (Molecular Medicine Program, National Institute of Child Health and Human Development, Bethesda, MD 20892, USA).

2.8.1. Vectors

The basic pGL4.10 vector (Promega, Cat No. E6651) does not contain any enhancer or promoter elements (Figure 2.1). The vector contains a multiple cloning region immediately upstream of the firefly luciferase reporter gene (luc2), into which the

FPN promoter was cloned in via XholI/HindIII sites, to drive expression of the reporter gene.

The MISSION 3'UTR Lentiviral Plasmid Vector pLSG_UTR_RenSP expresses an optimized luciferase reporter gene (RenSP) fused to a 3'UTR sequence (FPN) (Figure 2.2).

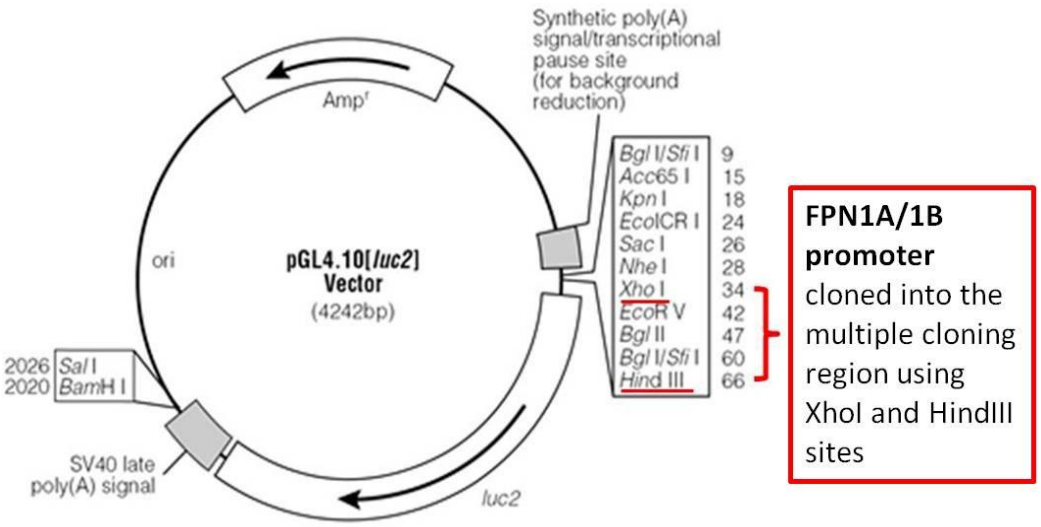


Figure 2.1 pGL4.10 luciferase reporter vector

The pGL4.10 vector was used to clone in FPN1A/FPN1B promoter in to the multiple cloning region. The promoter affects the expression of downstream firefly luciferase activity, allowing quantification of promoter activity.

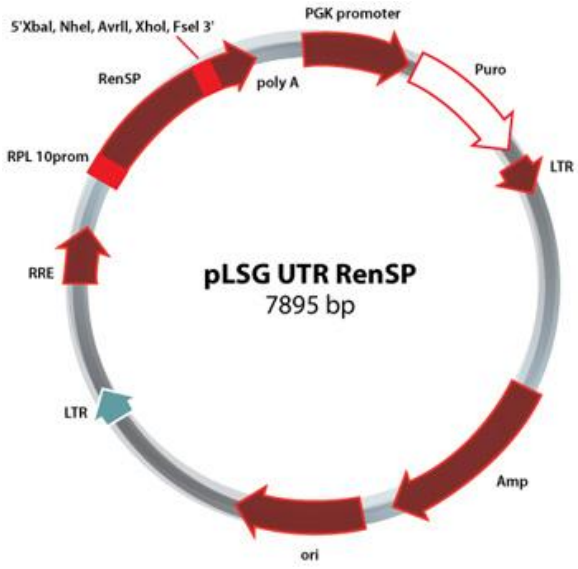


Figure 2.2 Lentiviral Plasmid Vector pLSG_UTR_RenSP

2.8.2. Plasmid transformation

The plasmids arrived on filter paper in an air-sealed plastic wallet. To extract the DNA, plasmids were cut from the filter paper and immersed in a corresponding Eppendorf of 200 µl nuclease-free water for 1 hour at room temperature. The concentration was measured on a Nanodrop spectrophotometer and the appropriate volume for 10 pg – 100 ng of DNA (typically 1 – 5 µl) was used to transform the plasmid into Escherichia coli (*E. coli*) competent cells using One Shot TOP10 Chemically Competent *E. coli* kit (Invitrogen C4040-03).

E.coli cells were thawed on ice from –80 °C for 30 min. The plasmid was added and mixed by gently flicking the tube several times and placed back on ice for 30 min. The mixture was then heat shocked at 42 °C for 30 seconds without shaking and placed immediately back on ice for 2 min. 250 µl room temperature SOC media (provided in

the kit) was added to each transformation and the cells were placed horizontally on a shaker at 225 rpm for 60 min at 37 °C. 25 µl of the reaction was spread onto pre-warmed Luria-Bertani (LB) agar plates containing 50 µg/ml carbenicillin and incubated at 37 °C overnight. This gave rise to single colonies that had successfully incorporated the plasmid and were able to survive by having selective antibiotic resistance.

A single colony was chosen, picked off the agar plate using a pipette tip and placed in 200 ml of autoclaved LB broth (4 g LB broth powder (Sigma, Cat No L3022-250G) in 200 ml water) with 50 µg/ml carbenicillin added at the end. The mixture was placed on a shaker at 200 rpm, 37 °C overnight. Cells were harvested the next day by centrifugation at 4 °C at 6000 x g for 15 min. To isolate the plasmid DNA, a QIAGEN Plasmid Midi Kit (QIAGEN, Cat No 12143) was used and manufacturer's protocol followed. Briefly, after centrifugation, the bacterial pellet was resuspended in 6 ml of cell lysis buffer P1 and then mixed with 6 ml of buffer P2. After vigorous mixing by inverting, it was left to stand for 5 min at room temperature. 6 ml of pre-chilled buffer P3 was added, thoroughly mixed and lysate transferred to a QIAfilter cartridge (provided in kit) where it was left to stand for 10 min. After which the lysate was filtered in to a pre-equilibrated HiSpeed midi tip. The flow-through was discarded and the tip washed with 20 ml buffer QC by gravity flow. DNA was eluted with 5 ml of buffer QC and the eluate collected in a 15 ml falcon tube. DNA was precipitated by adding 3.5 ml of room-temperature isopropanol to the tube, mixed and incubated at room temperature for 5 min. The eluate/isopropanol mixture was filtered through a QIAprecipitator and DNA washed with 2 ml of 70% ethanol. 1 ml of buffer TE was

added to a 5 ml syringe with the QIA precipitator attached and the DNA eluted in to a 1.5 ml collection tube.

The DNA obtained was quantified using the NanoDrop spectrophotometer and sent off for sequencing to DBS Genomics Durham University. The sequence received was viewed using FinchTV software and underwent nucleotide BLAST on online NCBI database (blast.ncbi.nlm.nih.gov/) to confirm the presence of FPN promoter sequence.

2.8.3. Transfection

Caco-2 cells were seeded onto 24 well plates until reaching 60 - 80% confluence (50,000 cells/well). After 24 hours, cells were transfected with empty pGL4 plasmid, FPN1A or FPN1B using FuGENE HD Transfection Reagent (Promega UK, Cat No E2311). A master mix was prepared for each plasmid (Table 2.8.1) and incubated at room temperature for 15 min before adding 25 µl to each well in a drop-wise manner.

Table 2.14 Fugene transfection master mix

Solution	Quantity
Serum-free DMEM	Up to 25 µl
Fugene	0.75 µl
Renilla	0.01 µg
Plasmid	0.2 µg

Fugene was added to serum-free DMEM and incubated at room temperature for 5 min after which plasmid DNA was added (DNA:Fugene = 1 µg:3 µl). After 15 min incubation at room temperature, 25 µl was added to each well.

Following transfection, cells were treated 24 hours later.

2.8.4. Dual luciferase reporter assay

The Dual-Luciferase Reporter Assay System (Promega UK, Cat No E1910) was used.

After treatment, media was removed and cells were washed twice with PBS. After adding 100 µl/well of passive lysis buffer, samples were incubated on a shaker for 15 min to ensure they were thoroughly lysed. 10 µl of cell lysate was added to 50 µl of LARII reagent (mixture of luciferase buffer and substrate as provided in the kit). The firefly luciferase signal was read with a GloMax 20/20 Luminometer (Promega, USA). After initial reading, Stop and Glo buffer, which consists of a firefly signal quencher and Renilla substrate, was added to the 1.5 ml Eppendorf and a second reading was taken for Renilla luciferase activity. Firefly luciferase activity was normalized against Renilla luciferase activity and the ratio calculated.

2.9. miRNA array

2.9.1. Sample preparation

Fully differentiated Caco-2 cells were either untreated (control) or treated with 10 µM quercetin for 24 hours. RNA was isolated using TRI Reagent as previously described. Samples for each treatment group were pooled together into 2 separate Eppendorf tubes and RNA concentration was measured on a NanoDrop spectrophotometer. Each pooled sample was diluted with nuclease free water to give a total RNA concentration of 500-1000 ng and made up to 8 µl.

2.9.2. Poly-A tailing and FlashTag Biotin HSR ligation

Keeping RNA on ice at all times, 2 µl RNA spike control oligonucleotides were added to each Eppendorf containing the pooled samples. The ATP mix was diluted in 1mM Tris 1:500 as directed by manufacturer's protocol before being added to the samples as shown in Table 2.15 to give a final volume of 15 µl per Eppendorf.

Table 2.15 Components needed for poly-A tailing

Component	Volume (µl) for 1 sample
10X Reaction Buffer	1.5
25mM MnCl2	1.5
Diluted ATP mix	1.0
PAP enzyme	1.0

Each sample was gently mixed by hand, microfuged and then placed on a 37ºC heat block for 15 min. After incubation, the samples were microfuged briefly and placed on ice. 4 µl 5X FlashTag Biotin HSR Ligation mix was added, followed by 2 µl of T4 DNA ligase to each of the samples. After gentle mixing and microfuge, the samples were incubated at room temperature for 30 min. The reaction was stopped by adding 2.5 µl HSR stop solution, giving a final volume of 23.5 µl. 21.5 µl of this was used for the next stage. The mixture may be stored for 6 hours on ice or up to 2 weeks at -20ºC prior to hybridization on an Affymetrix GeneChip.

2.9.3. Hybridization

Table 2.16 Hybridization cocktail

Component	Volume (µl) for 1 sample
2X Hybridization mix	50
27.5% Formamide	15
DMSO	10
20X Eukaryotic Hybridization controls	5
Control oligonucleotide B2, 3nM	1.7

All components listed in Table 2.8.3 were bought to room temperature (20X Eukaryotic Hybridization Controls was heated for 5 min at 65ºC) and added in the order listed to 21.5 µl of the biotin-labelled samples from section 2.9.2. The total volume was 103.2 µl for each sample. The mixture was incubated at 99ºC for 5 min and then at 45ºC for 5 min. 100 µl of each sample was aspirated and injected into a labelled, corresponding array. Care was taken to prevent evaporation or leaks. The arrays were placed on to trays and placed into pre-heated hybridization oven where they were incubated at 48ºC for 16 hours at 60 rpm.

2.9.4. Washing and staining

The arrays were removed from the oven after their incubation period and hybridization cocktail removed from each array. These may be stored at -80ºC for future use. Each array was then completely filled with Array Holding Buffer and left to equilibrate to room temperature.

600 µl stain cocktail 1; 600 µl stain cocktail 2 and 800 µl array holding buffer were loaded on to sample holders on the fluidics station as directed by manufacturer's protocol. The programme was set to wash and stain with Fluidics station 450 using fluidics script FS450_0003. The arrays were checked for air bubbles and dust on the array glass surface before scanning.

2.9.5. Analysis

miRNA QC Tool Software was used for data summarisation, normalisation and inspecting quality control. After miRNA QC Tool analysis was run, data was exported to Microsoft Excel software for further analysis of fold changes of individual miRNAs. Selected miRNA showing significant changes were validated by q-PCR.

2.10. miRNA validation

miRNAs were validated using miRCURY LNA™ Universal RT microRNA PCR kit (Cat No 203301). RNA samples from quercetin-treated Caco-2 cells was diluted to 5 ng/µl for reverse transcription. Following the manufacturer's protocol, a working solution was made (Table 2.10.1) and dispensed into nuclease free PCR Eppendorf tubes. After brief centrifugation, tubes were placed in a thermal cycler in the following conditions: incubation; 42ºC for 60 min, heat inactivate reverse transcriptase; 95ºC for 5 min and immediately cool to 4ºC for use or short-term storage.

2.10.1. Real-time PCR

cDNA template was diluted 1:80 in nuclease free-water (e.g. add 395µl water to each 5 µl of reaction). Primer:master mix working solution was prepared as in Table 2.10.2 and added to well of PCR plate. Plate was sealed and centrifuged (1500xg for 1 minute). Real-time PCR amplification performed according to Table 2.10.3.

Table 2.17 Reverse transcription setup per reaction

Reagent	Volume (µl)/reaction
5 × Reaction Buffer	2.0
Nuclease-free H2O	4.5
Enzyme mix	1.0
Synthetic RNA spike in (optional; replace with H2O if omitted)	0.5
Template total RNA (5 ng/µl)	2.0
Total volume	**10.0**

Table 2.18 Components of qPCR reaction plate

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix	12.5 µl	1x
Forward Primer	1.25 µl	0.5 µM
Reverse Primer	1.25 µl	0.5 µM
RNase-free water	10 µl	----
Template (cDNA or Standard)	1 µl	~1000 ng/reaction
Total volume	25 µl	----

Table 2.19 qPCR cycling parameters for ABI Prism 7500 fast real-time cycler

Cycles	Temp (°C)	Time	Comment
1	95	10 min	Polymerase activation/denaturation
40	95 60	10 sec 1 min	Amplification

Data was analysed using ABI Prism software. Expression data were normalized to control SNORD49a expression.

Table 2.20 miRNA target sequence

miRNA	mRNA sequence
hsa-miR-485-3p	GUCAUACACGGCUCUCCUCUCU
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-17-3p	ACUGCAGUGAAGGCACUUGUAG
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7d-3p	CUAUACGACCUGCUGCCUUUCU
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
hsa-miR-92b-5p	AGGGACGGGACGCGGUGCAGUG

2.11. Data analysis

GraphPad Prism 5.0 (GraphPad software) was used for statistical analysis. Data (Mean ± SEM) were analysed by one-way ANOVA followed by Dunnett's post hoc test, Student's unpaired t-test or Kruskal-Wallis test where appropriate. Significance was considered at $p<0.05$.

3. Results I

Caco-2 intestinal cell line

3.1. Introduction

In recent years, several dietary factors that affect non-haem iron absorption from foods have been identified. Human studies usually involve single test meals extrinsically tagged with radioactive iron (Cook et al. 1972). Through use of this method, it has been shown that single meals containing high amounts of polyphenols inhibit non-haem iron bioavailability (Samman et al. 2001, Reddy et al. 2000). Although this method allows investigators to control a number of variables, there are questions over its relevance. There appear to be discrepancies between single meal studies and long-term studies where a more varied diet is in place. For example a pronounced inhibition of iron by polyphenols has been observed in single-meal human studies but this effect has not been noted in longitudinal studies where consumption of high levels of polyphenols occurs over a long period of time. In a review of the related literature specific to polyphenols in black tea on iron absorption, it was suggested that moderate tea drinking, and thus a consistent intake of polyphenols was unlikely to have an adverse effect on iron status in healthy adults (Nelson and Poulter 2004).

A characteristic feature of quercetin and its metabolites is its slow elimination with reported half-lives ranging from 11 to 28 hours (Manach et al. 2005) which means repeated and regular intakes could lead to plasma accumulation. This long-term effect would cause plasma levels of quercetin to exceed those achieved in single meal studies.

The aim of this first section was to bridge the existing gap between short-term and long-term effects of polyphenols on intestinal non-haem iron absorption. Quercetin was chosen as the polyphenol of choice due to its dominance in the diet and the Caco-2 cell model was used.

Caco-2 cells originate from human colon adenocarcinoma cells (Fogh et al. 1977) and are frequently used as an *in vitro* intestinal cell model. As cells differentiate, they polarize to develop apical and basolateral membrane, exhibiting a clearly defined apical brush border and tight intracellular junctions (Peterson and Mooseker 1992). Upon confluence, they form a monolayer, allowing transport of nutrients from the apical to the basolateral membrane making it a useful model to study intestinal nutrient and chemical transport. The TC7 clone used in this study is derived from a higher passage (p198) (Chantret et al. 1994) and is more morphologically characteristic of enterocytes and its transporters than the parental cell line.

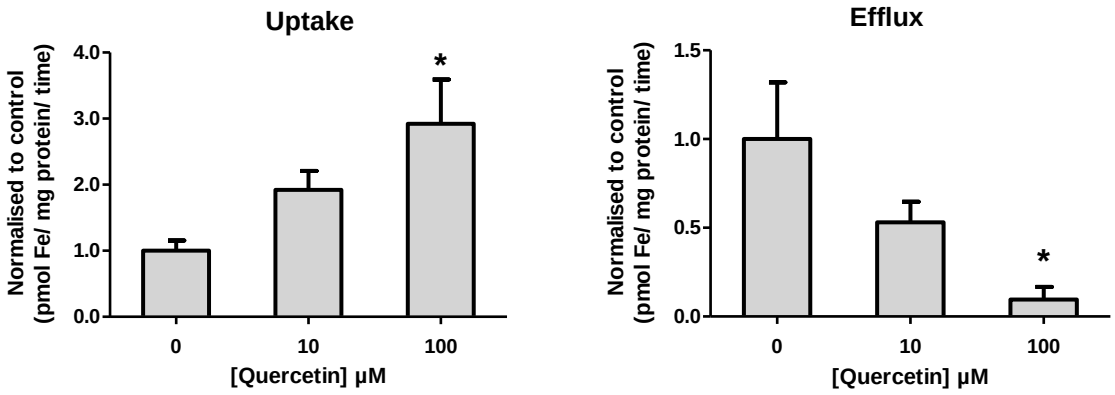
As the small intestine is the primary site of iron absorption, using an *in vitro* model allowed us to inspect intestinal iron transport in more detail and make changes in treatments, timings and conditions more readily.

3.2. Quercetin decreased ^{55}Fe efflux

Caco-2 cells were grown on Transwell inserts, with the use of differential pH to achieve transepithelial gradient and recognition of apical (pH 6.5) and basal (pH 7.5) membranes. For acute effects, quercetin was added along with ^{55}Fe to the apical compartment and incubated for 20 minutes for uptake and 120 minutes for transport. For chronic effects, cells were pre-incubated with quercetin for 24 hours, after which the media was replaced with buffer containing ^{55}Fe alone and subjected to uptake (20 min) and transport (120 min) measurements. Cell lysates were used to measure protein concentration using the Bio-Rad method previously described and uptake and transport measurements were corrected to their corresponding protein levels.

In the acute setting, quercetin caused a significant dose-dependent increase in iron uptake ($p<0.05$; Figure 3.1). However, during chronic conditions, where cells were exposed to quercetin for 24 hours, this effect was largely abrogated with no significant differences being noted. Conversely, quercetin caused a dose-dependent decrease in iron efflux in both the acute and the chronic setting ($p<0.05$; Figure 3.1 right hand panels).

A) Acute



B) Chronic

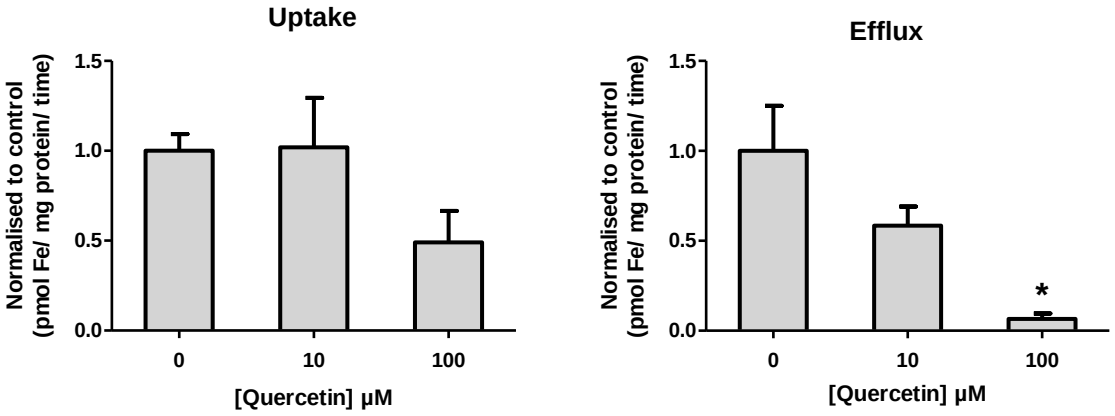


Figure 3.1 Effect of quercetin on ^{55}Fe bioavailability in Caco-2 cells

Fully differentiated Caco-2 cells grown on Transwell inserts were treated with 1, 10 or 100 µM quercetin for (A) 20 min or (B) 24 hours. Apical uptake of iron was measured after 20 min and basolateral release of iron was measured after 120 min. All data was normalised to protein concentration. Data analysed by one-way ANOVA and Dunnett's posthoc test compared to untreated control group; significance was determined at (*) $p<0.05$. Data presented as mean ± SEM; n = 6.

3.3. Quercetin induces down regulation of basolateral iron transporter mRNA expressions

With the previous functional experiments showing that quercetin decreased iron efflux rather than iron uptake, we next investigated the molecular mechanism underpinning these effects. We started by looking at messenger RNA (mRNA) expression of genes controlling intestinal iron absorption. In particular this included duodenal cytochrome b (Dcytb) and divalent metal transporter 1 (DMT1) found on the apical membrane, responsible for iron uptake and ferroprotin and hephaestin which are found on the basolateral membrane responsible for transporting iron out of the cell.

Caco-2 cells were treated with quercetin (0, 0.1, 1, 10 and 100 µM) 24 hours, followed by RNA isolation and quantitative-PCR to measure changes in expression. Dcytb mRNA showed no significant change with quercetin treatment; however an increasing trend could be noted (Figure 3.2). DMT1 expression was not significantly altered by exposure to any of the quercetin concentrations.

Quercetin caused downregulation of ferroportin, significant at 10 µM (0.47 ± 0.17; $p<0.05$) and 100 µM (0.12 ± 0.04; $p<0.001$; Figure 3.2). Its partner hephaestin also exhibited a dose dependent-decrease, again significant at 10 µM (0.36 ± 0.09; $p<0.001$) and 100 µM (0.29 ± 0.04; $p<0.001$). Therefore it was understood that the gene expression of the apical transporters remained unaffected by quercetin treatment whereas the basolateral transporters were down regulated.

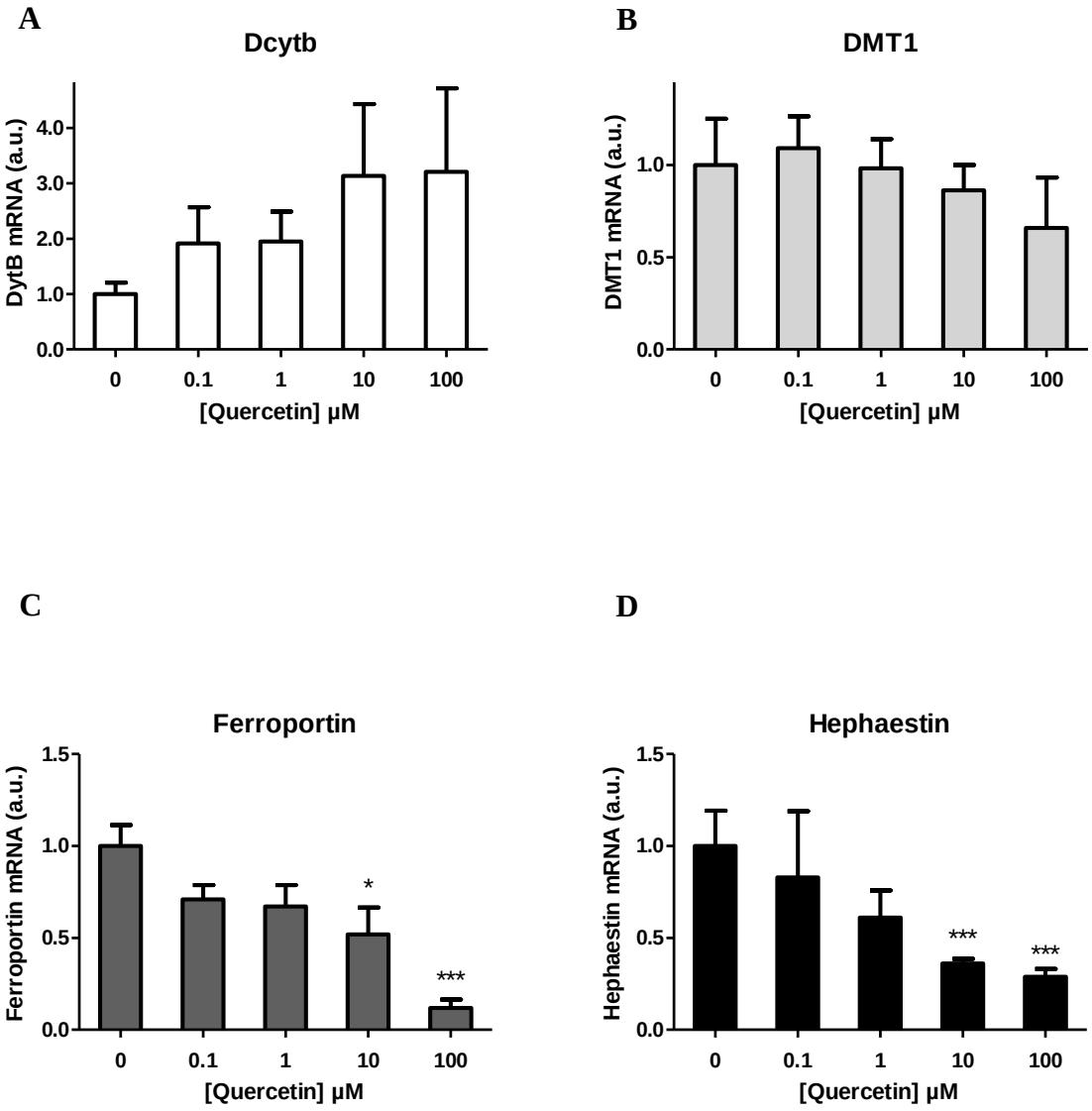


Figure 3.2 Dose-response effect of quercetin on mRNA expression of four important genes involved in intestinal iron transport

Caco-2 cells were treated with quercetin (0-100 µM) for 24 hours. Changes in mRNA expression of (**A**) Dcytb n=9; (**B**) DMT1 n=9; (**C**); ferroportin n=14 (100 µM n=7) and (**D**) hephaestin n=8 (100 µM n=5) were measured by q-PCR. Data was normalised to control. Data analysed by one-way ANOVA and Dunnett's posthoc test compared to untreated control. Significance: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data presented as mean ± SEM.

3.4. Quercetin significantly decreases ferroportin protein expression

To determine whether there were any functional consequences of ferroportin mRNA down regulation, we proceeded to examine the effects of quercetin on the protein expression of the two main iron transporters DMT1 and ferroportin.

Cells were treated with quercetin (0 - 10 μM) for 24 hours. Thereafter cells were lysed and protein isolated and quantified. β-actin was measured as a housekeeping control. Films of western blots were scanned and semi-quantified using ImageJ software.

The protein expression of DMT1 showed no change following exposure to quercetin. Ferroportin expression however, was significantly reduced in the presence of 10 μM quercetin ($p<0.05$; Figure 3.3).

This protein data validated that quercetin does not affect DMT1, either at the gene or protein level, but quercetin does decrease ferroportin mRNA expression and this effect is translated into its protein expression.

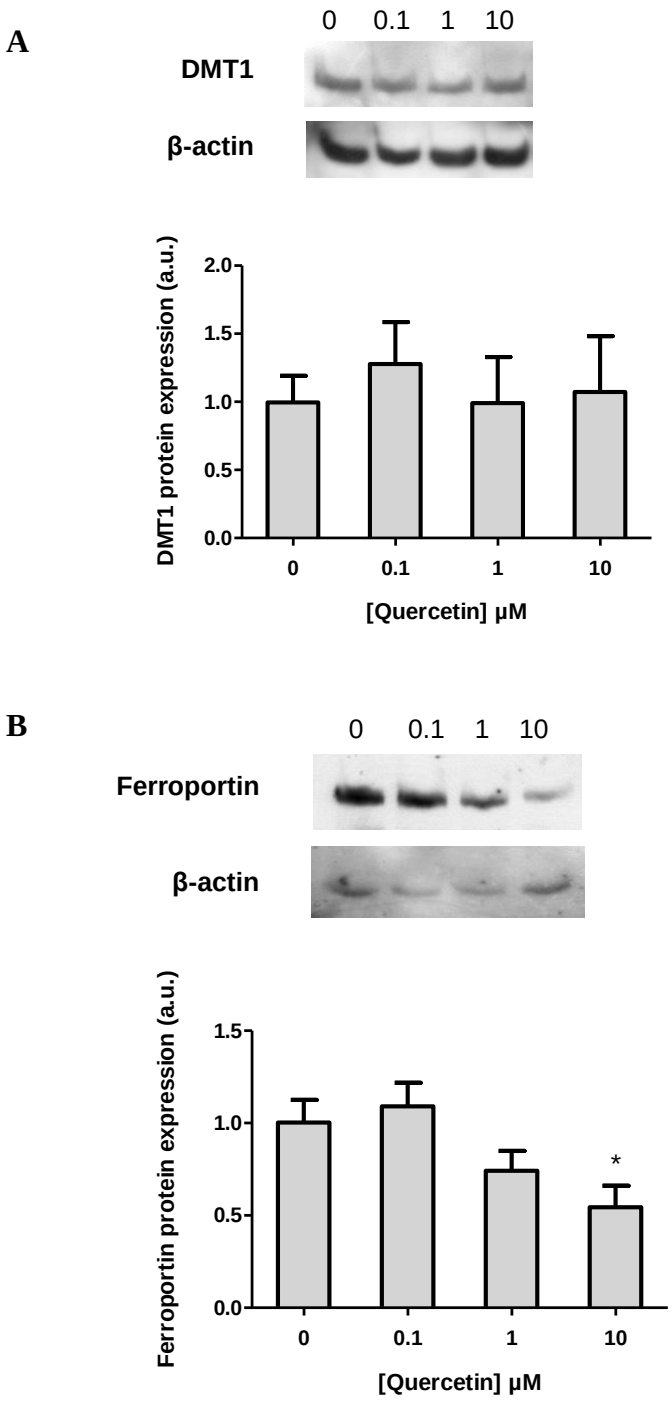


Figure 3.3 Quercetin had no effect on DMT1 protein levels but decreased ferroportin protein levels

Caco-2 cells were treated with quercetin (0-10 µM) for 24 hours followed by cell lysis and protein isolation. A representative western blotting band for each treatment is shown for (**A**) DMT1 n=6 and (**B**) ferroportin n=9. Blots were semi-quantified using Image J software. All results were normalised to β-actin and control group. Data analysed by one-way ANOVA and t-test compared to untreated control group. Data presented as mean ± SEM.

3.5. Effects of quercetin on ferritin and IRP2

Next we addressed the mechanism by which quercetin affects ferroportin expression. As quercetin is a known iron-chelator, we investigated whether quercetin was affecting the iron status of cells. We used ferritin and IRP2 as biomarkers of cellular iron content.

3.5.1. Quercetin decreased ferritin protein levels

Caco-2 cells were treated with 0, 0.1, 1, 10 μM quercetin for 24 hours. Cells were lysed and protein isolated and quantified. Changes in ferritin were measured using a colorimetric ELISA assay and readings were normalised to corresponding protein levels (Spectro-ferritin kit ATI Atlas Ltd UK, Cat No S-22).

We found that with increasing concentrations of quercetin (0-10 μM), ferritin levels decreased, and were significantly lower than control values in cells exposed to 1 and 10 μM quercetin ($p<0.05$; Figure 3.4).

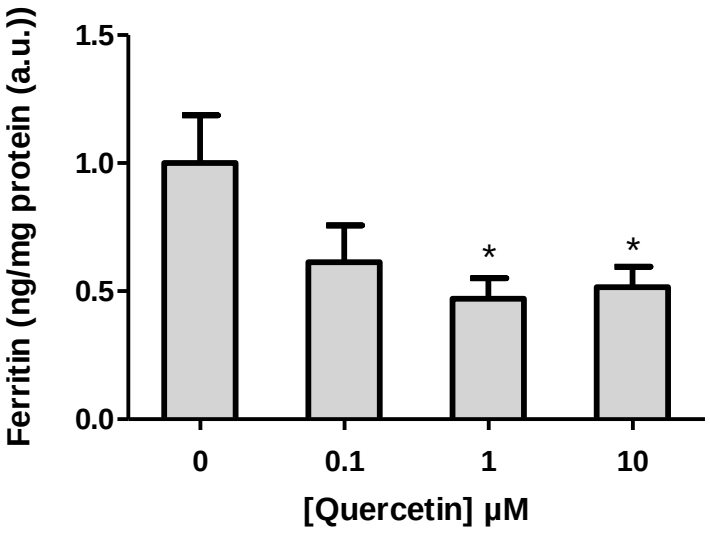


Figure 3.4 Quercetin decreased ferritin protein levels

Caco-2 cells were treated with quercetin (0-10 µM) for 24 hours followed by cell lysis and protein isolation. Ferritn was quantified using a colorimetric ELISA kit. Results were normalised to corresponding protein levels and control group. Data analysed by one-way ANOVA and Dunnett's post-hoc test compared to control group. N=15. Data presented as mean ± SEM.

3.5.2. Effects of quercetin and FAC on IRP2 protein levels

Caco-2 cells were treated with 0 - 100 µM quercetin, 50 µM ferric ammonium citrate (FAC) or quercetin and FAC combined for 24 hours. Cells were lysed and protein isolated and quantified. IRP2 protein was measured by the western blotting detection system.

Quercetin at 100 µM produced a modest non-significant increase of IRP2 level (Figure 3.5), whereas 50 µM ferric ammonium citrate (FAC) produced a slight decrease which could be recovered by 10 µM quercetin. FAC (50 µM) combined with 100 µM quercetin caused a significant increase of IRP2 ($p<0.01$).

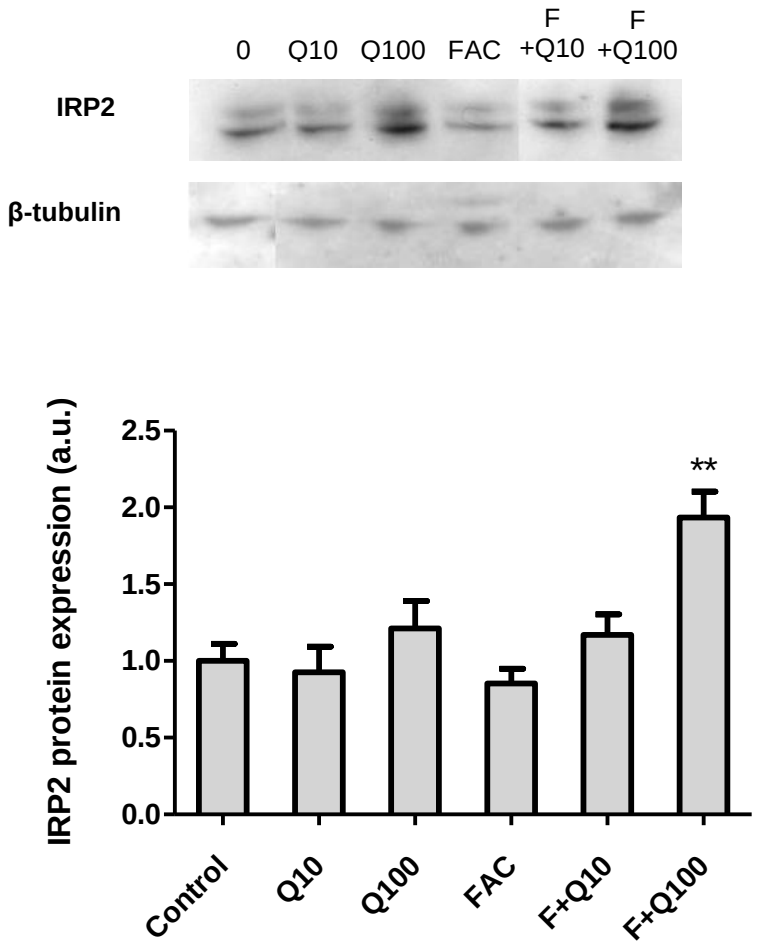


Figure 3.5 Effect of quercetin and FAC on IRP2 protein levels

Caco-2 cells were treated with quercetin (0-100 µM) or FAC (50 µM) or in combination for 24 hours. A representative band is shown. Blots were semi-quantified using Image J software. All results were normalised to β-tubulin and control group. Data analysed by one-way ANOVA and Dunnett's post-hoc test compared to control group. N=8. Data presented as mean ± SEM.

3.5.3. Quercetin increased nuclear Nrf2 protein levels

Quercetin is a powerful free radical scavenger, able to prevent or delay conditions which favour cellular oxidative stress. Nrf2 is a transcription factor that functions as a key inducer of antioxidant and detoxifying enzymes. Nrf2 regulatory effect on FPN and contribution to the cellular antioxidant defense system has not yet been looked at in intestinal cells. We postulated that quercetin may regulate intestinal ferroportin expression via Nrf2.

Fully differentiated intestinal Caco-2 cells were treated with different concentrations of quercetin 0 – 100 µM (encompassing those concentrations used in the above studies) for 24 hours, followed by protein isolation. We looked at Nrf2 proteins in both cytosolic and nuclear preparations. To separate and extract each fraction NE-PER Nuclear and cytoplasmic Extraction Reagent (thermo Scientific Cat No 78833) kit was used.

We found that there was no change in cytosolic levels of Nrf2 with quercetin treatment (Figure 3.6). In contrast, the nuclear component showed a dose-dependent increase of Nfr2 with increasing concentrations of quercetin, significant at 100 µM ($p<0.001$).

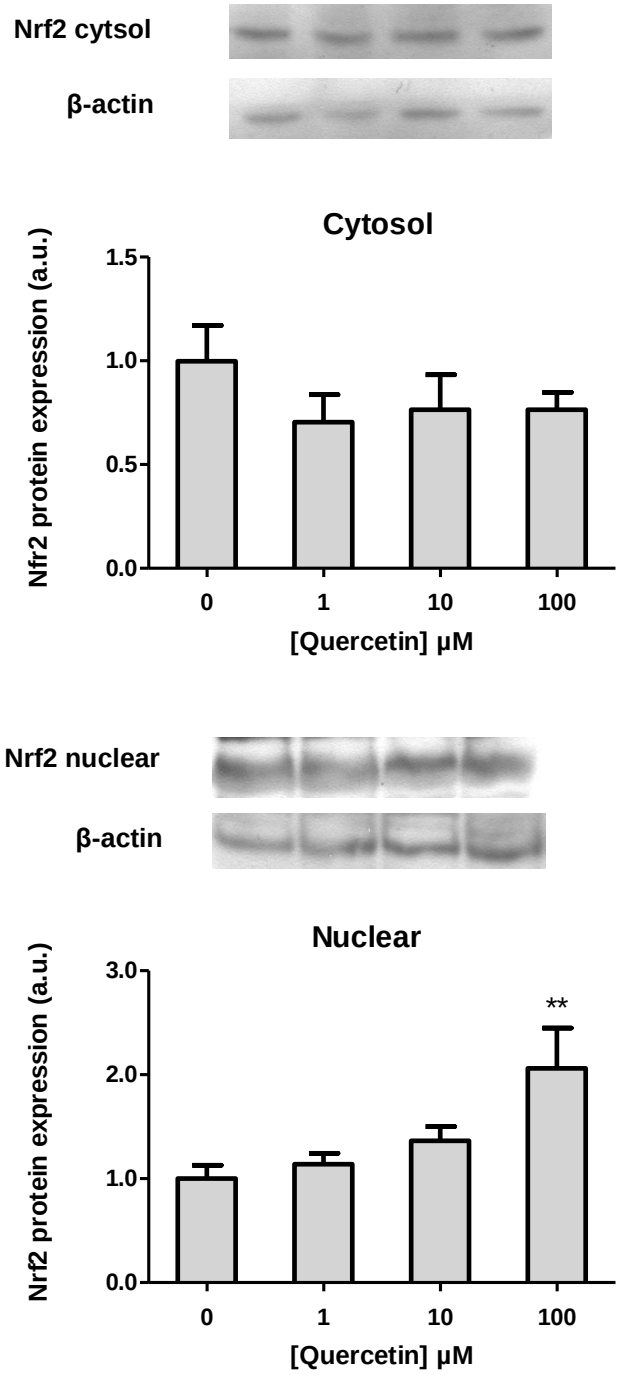


Figure 3.6 Quercetin increased nuclear Nrf2 protein levels

Caco-2 cells were treated with quercetin (0-10 µM) for 24 hours followed by extraction of cytosolic and nuclear fractions. A representative band is shown for (**A**) cystosolic and (**B**) nuclear fractions. Blots were semi-quantified using Image J software. All results were normalised to β-actin and control group. Data analysed by one-way ANOVA and Dunnett's post-hoc test compared to untreated control group. N=8. Data presented as mean ± SEM.

3.6. Erythropoietin (Epo)

Erythropoietin (Epo) is a glycoprotein hormone produced by the kidneys in response to hypoxia, to meet body demands of increased erythropoiesis. There has been work to show that Epo may have direct effects on intestinal iron absorption (Srai et al. 2010) as Epo treatment induced up-regulation of iron transporters DMT1 and ferroportin and as such increased iron transport across Caco-2 monolayers. Epo activates many different signalling pathways to regulate iron homeostasis. Of particular interest for this study was the PI3K (phosphatidylinositol 3-Kinase) pathway which is an important modulator of gene expression and regulates a number of cellular responses. Specific inhibitors of the PI3K pathway have structurally similar properties to quercetin. We therefore wondered whether quercetin might influence Epo stimulation of iron transporter expression via as similar mechanism.

3.6.1. Effects of Epo and PI3K inhibitor

Caco-2 cells were grown on 12-well plates and treated for 24 hours on day 16 of cell culture growth. Cells were exposed to either Epo (1 Unit/ml), quercetin (10 or 100 µM) and Epo in combination, PI3K inhibitor (20 µM: LY (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), and Epo and PI3K inhibitor in combination. The four main genes in iron transport were looked at.

Dcytb

Dcytb mRNA expression was not significantly altered by treatment with Epo (Figure 3.7A). Co-incubation of Epo and quercetin (10 and 100 µM) as well as Epo and PI3K inhibitor also had no significant effect.

DMT1

Treatment with Epo non-significantly increased (2.2 fold from control) DMT1 expression (Figure 3.7B). This rise caused by Epo was reduced almost back to control levels with addition of either 10 or 100 µM quercetin. PI3K inhibitor decreased DMT1 expression below basal level.

Ferroportin

Epo significantly increased ferroportin mRNA expression ($p<0.05$; Figure 3.7C). Co-incubation of Epo with quercetin returned ferroportin expression back to basal levels. A similar effect was seen when Epo was incubated with PI3K inhibitor.

Hephaestin

Epo has no effect on hephaestin expression (Figure 3.7D) and treatment with Epo + quercetin (10 and 100 µM) did not alter hephaestin levels compared with control. PI3K inhibitor reduced hephaestin expression but this was not significant.

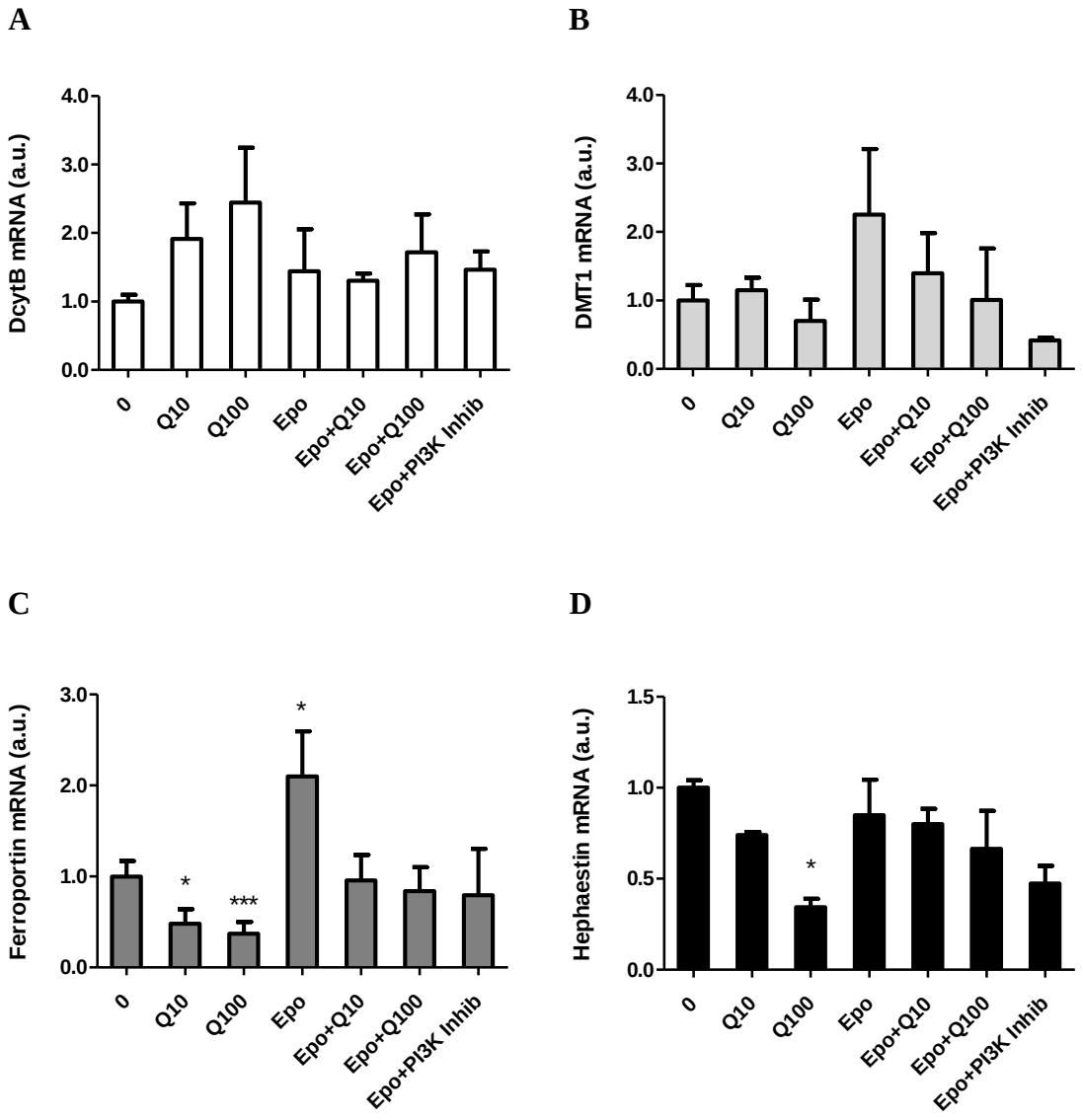


Figure 3.7 Effects of Epo and PI3K inhibitor on genes involved in iron transport

Caco-2 cells were treated with quercetin (10 or 100 µM), Epo (1 U/ml) or PI3K inhibitor (20 µM) alone or in combination for 24 hours. Changes in mRNA expression of (**A**) Dcytb n=6; (**B**) DMT1 n=8; (**C**); ferroportin n=10 and (**D**) hephaestin n=4. Data was normalised to control. Data analysed by one-way ANOVA and Dunnett's post-hoc test compared to untreated control. Significance: * $p<0.05$; ** $p<0.01$. Data presented as mean ± SEM.

3.7. Ferroportin promoter

As a result of alternative splicing there are two possible isoforms of ferroportin that can arise – one which has an IRE in the 5' UTR named FPN1A and one which lacks this IRE named FPN1B (Zhang et al. 2009). To investigate whether quercetin affects the ferroportin promoter and thereby ferroportin gene expression, both these transcripts were transiently transfected in Caco-2 cells.

FPN1A promoter is 895 base pairs (bp) in size starting 845 bp before the transcription initiation site (just after exon 1b) and 50 bp after the initiation site. FPN1B promoter is 1050 bp starting 1000 bp before the FPN1B transcription initiation site to 50 bp after the initiation site. These promoters were cloned into a pGL4 luciferase vector via XhoI/Hind III sites.

3.7.1. Control studies for transfection efficiency and determining quercetin concentration

First, transfection efficiency was measured. This was to ensure that the promoter was correctly inserted and driving firefly luciferase expression. FPN1A caused over a 90-fold increase in luciferase activity compared to an empty, promoter-less pGL4 vector (Figure 3.8) ($p<0.0001$). FPN1B caused a significant 4-fold increase compared to empty pGL4 vector ($p<0.0001$).

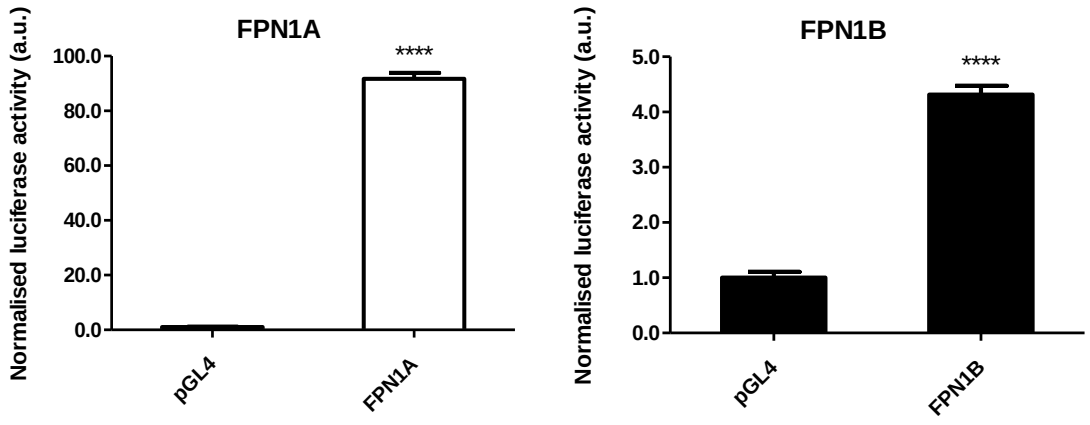


Figure 3.8 Comparison of promoter activities between empty vector and FPN pGL4
Caco-2 cells were incubated for 24 hours after transfection and harvested for Luciferase assay. Data analysed by t-test compared to empty pGL4 vector. Significance: **** $p<0.0001$; n=3; data presented as mean ± SEM.

Next we looked at the optimal concentration of quercetin to use in our studies. There was no significant difference in luciferase activity between empty pGL4 and pGL4 co-treated with 10 µM of quercetin (Figure 3.9). However, there was a significant decrease caused by 100 µM quercetin ($p<0.05$). Therefore 100 µM quercetin concentration was omitted from further use in this particular experiment.

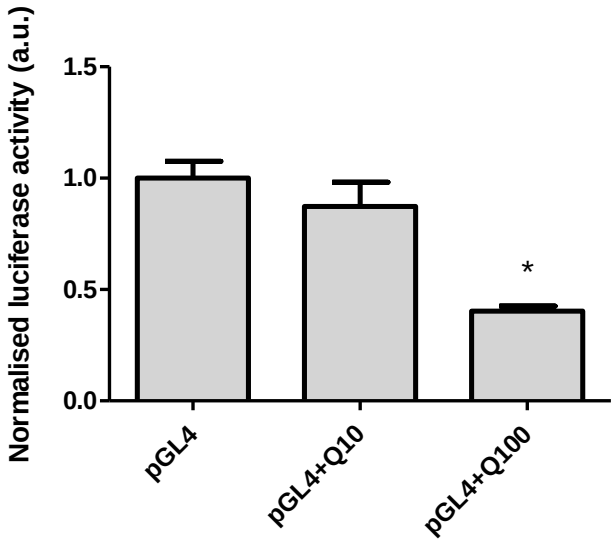


Figure 3.9 Quercetin 100 µM affects basal luciferase activity

Caco-2 cells were incubated 24 hours after transfection and treated with quercetin (10 or 100 µM) for a further 24 hours. Data analysed by one-way ANOVA and Dunnett's post-hoc test compared to pGL4 alone. Significance: * $p<0.05$; n=3. Data presented as mean ± SEM.

3.7.2. Effects of iron and quercetin on FPN1A and FPN1B promoter

To examine the response of the different promoter constructs to iron loading and iron depletion, Caco-2 cells were transiently transfected with the promoter-containing plasmids for 24 hours. Media was then replaced and cells were treated with quercetin (1 or 10 µM), FAC (50 µM ferric ammonium citrate) or FAC and quercetin (10 µM) combined.

There was no significant effect of quercetin treatment on either FPN1A or FPN1B promoter activity (Figure 3.10). Addition of FAC alone and together with quercetin also had no effect on promoter activities.

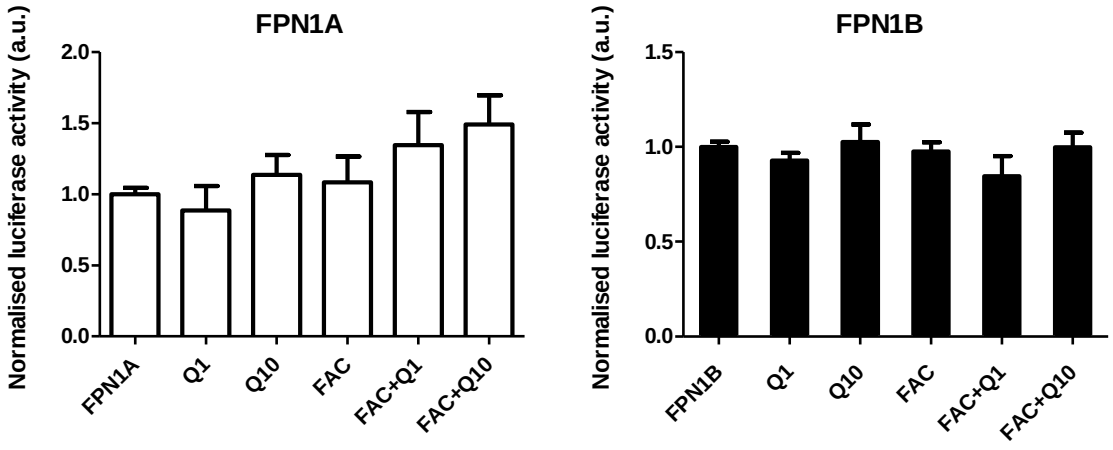


Figure 3.10 Quercetin and FAC had no affect on FPN1A/1B promoter activities

Caco-2 cells were incubated 24 hours after transfection and treated with quercetin (1 or 10 µM), DFO (100 µM) and/or FAC (50 µM) for a further 24 hours. Data was normalised to untreated transfection control (either FPN1A or FPN1B alone). Data analysed by one-way ANOVA and Dunnett's post-hoc test. Significance: ** $p<0.01$; n=6. Data presented as mean ± SEM.

3.7.3. Effects of Epo on FPN1A and FPN1B promoter

As Epo was previously shown to stimulate FPN mRNA expression we wanted to see what effect it would have on the ferroportin promoter activity and whether quercetin (10 µM) would affect the Epo response.

Epo had no effect on FPN1A activity but significantly increased FPN1B promoter activity ($p<0.01$; Figure 3.11). Co-treatment with quercetin did not diminish the response to Epo ($P<0.01$).

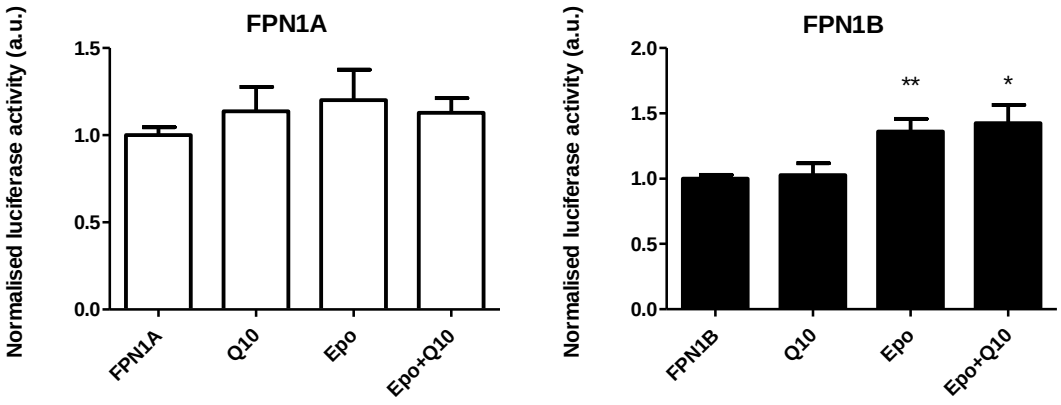


Figure 3.11 Epo increased FPN1B activity but had no effect on FPN1A

Caco-2 cells were incubated 24 hours after transfection and treated with quercetin (10 µM), Epo (1U/ml) or Epo + quercetin for a further 24 hours. Data was normalised to untreated transfection control (either FPN1A or FPN1B alone). Data analysed by one-way ANOVA and Dunnett's post-hoc test. Significance: * $p<0.05$ **$p<0.01$; N=3 (FPN1A, FPN1B, Q10 and Epo n=6). Data presented as mean ± SEM.

3.7.4. Effects of HIF1α and HIF2α inhibitors on promoter activity

Hypoxia inducible factors (HIFs) are transcription factors that can regulate the transcription of ferroportin. They are formed by an oxygen sensitive α subunit (HIF1α/HIF2α) and a constitutively expressed β subunit. To examine the effects of HIFs on basal ferroportin promoter activity, specific HIF1α (20 μM) and HIF2α (10 μM) inhibitors were used to block this oxygen-sensitive pathway. HIF inhibitors were also co-incubated with quercetin (10 μM).

FPN1A

There was no significant effect of either the HIF1α or the HIF2α inhibitor on FPN1A promoter activity (Figure 3.12). Co-incubation of HIF1α inhibitor with quercetin (10 μM) significantly decreased FPN1A promoter activity ($p<0.01$) whereas no significant effect of quercetin was noted with the HIF2α inhibitor.

FPN1B

Neither HIF1α inhibitor nor HIF2α inhibitor had an effect on FPN1B promoter activity (Figure 3.13). In contrast to FPN1A, co-incubation of either HIF inhibitor with quercetin (10 μM) had no effect on FPN1B. This suggests that HIFs use FPN1A promoter rather than FPN1B to exert their regulatory effects.

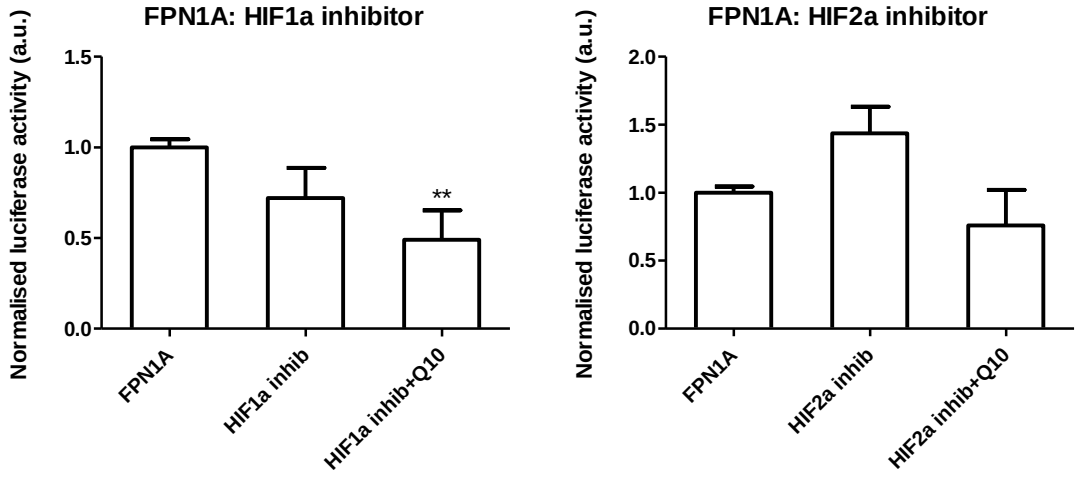


Figure 3.12 Co-treatment of HIF1α inhibitor and quercetin decreased FPN1A activity

Caco-2 cells were incubated for 24 hours after transfection and treated with HIF1α inhibitor (20 μM), HIF2α inhibitor (10 μM) either alone or together with quercetin (10 μM) for 24 hours. Data was normalised to untreated control (FPN1A alone without any treatment). Data analysed by one-way ANOVA and Dunnett's post-hoc test. Significance: **p<0.01 ***p<0.001; N=6; Data presented as mean ± SEM.

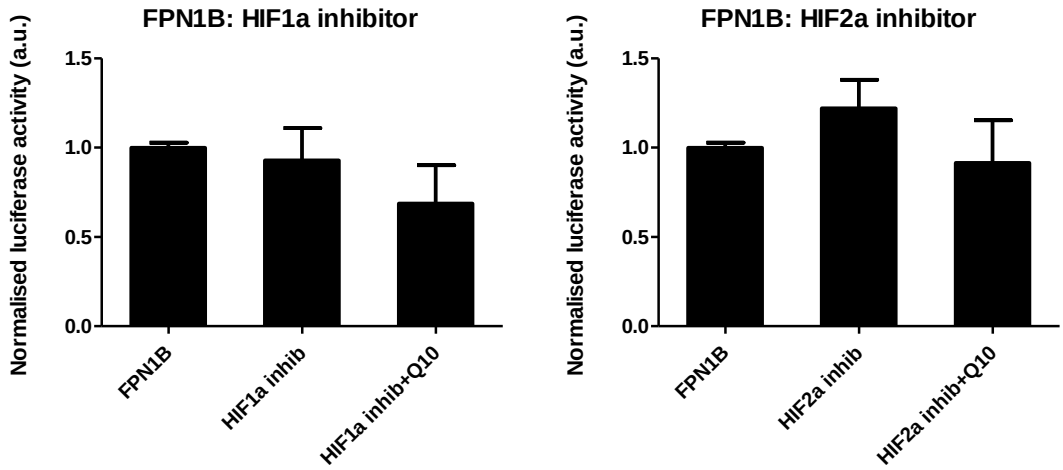


Figure 3.13 HIF1α/ HIF2α inhibitors have no effect on FPN1B promoter activity

Caco-2 cells were incubated for 24 hours after transfection and treated with HIF1α inhibitor (20 μM), HIF2α inhibitor (10 μM) either alone or together with quercetin (10 μM) for 24 hours. Data was normalised to untreated control (FPN1A alone without any treatment). Data analysed by one-way ANOVA and Dunnett's post-hoc test. Significance: **p<0.01 ***p<0.001; N=6; Data presented as mean ± SEM.

3.7.5. Effect of quercetin on FPN1A and FPN1B mRNA expression

To investigate whether quercetin was affecting one transcript over another, Caco-2 cells were treated with increasing doses of quercetin (0, 1, 10, 100 µM). Changes in gene expression were measured by q-PCR using specific primers for FPN1A and FPN1B.

FPN1A exhibited a dose-dependent decrease with increased quercetin concentrations (100 µM: 0.3 ± 0.06 $p<0.05$; Figure 3.14). On the other hand, quercetin increased expression of FPN1B, significant at 10 and 100 µM concentrations ($p<0.05$).

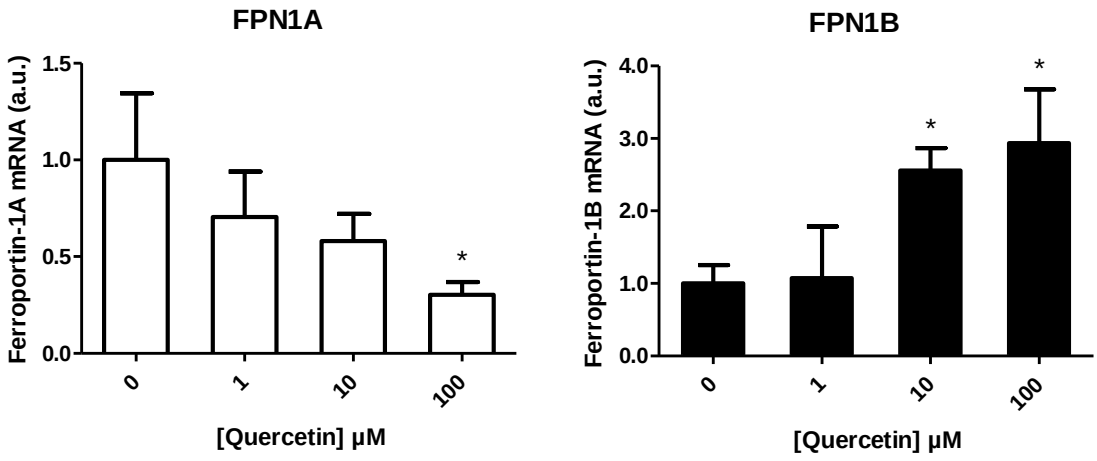


Figure 3.14 Quercetin decreased FPN1A mRNA and increased FPN1B mRNA

Caco-2 cells were treated with quercetin (0-100 µM) for 24 hours. Changes in mRNA expression of FPN1A (n=9) or FPN1B (n=4) were measured by q-PCR. Data was normalised to housekeeping 18S and control. Data analysed by one-way ANOVA and Dunnett's posthoc test compared to untreated control. Significance *$p<0.05$. Data presented as mean ± SEM.

3.8. MicroRNA (miRNA)

Given the importance of FPN, it is not surprising that its regulation occurs at multiple levels. MicroRNAs (miRNAs) present a novel mechanism of post-transcriptional regulation, independent of IRP/IRE regulation that occurs at the 5'UTR of FPN. miRNAs are small endogenous non-coding RNA that bind to complementary sequences in the 3'UTR of target mRNA, usually resulting in mRNA degradation or translational repression.

Iron status is a factor that appears to affect many miRNAs. A recently published paper found that miR-485-3p in particular, may regulate FPN by directly targeting FPN 3'UTR (Sangokoya et al. 2013). This microRNA was induced during iron deficiency and repressed FPN protein expression in HepG2 (hepatocyte) and K562 (human erythroid) cell lines. This provided evidence that miRNAs are capable of regulating FPN expression.

Having looked at FPN 5'UTR, it was interesting to see what effect quercetin would have on FPN 3'UTR. We began this investigation by carrying out miRNA arrays on quercetin-treated samples to screen for quercetin-induced miRNA changes in Caco-2 cells. We identified a number of miRNAs that were regulated by quercetin. Table 3.1 shows those that were upregulated by at least 1.5-fold as compared to control.

Table 3.1 Fold changes of miRNA with quercetin treatment.

miRNA name	Fold changes
hsa-miR-1285_st	1.50925
hsa-miR-181b_st	1.51684
hsa-miR-17-star_st	1.53885
hsa-miR-138_st	1.53899
hsa-miR-362-3p_st	1.54032
hsa-miR-183-star_st	1.55294
hsa-miR-342-3p_st	1.55326
hsa-miR-330-3p_st	1.55861
hsa-miR-150-star_st	1.56930
hsa-miR-29b-2-star_st	1.57430
hsa-miR-188-5p_st	1.58094
hsa-miR-130a_st	1.58734
hsa-miR-193b-star_st	1.59014
hsa-miR-30c-1-star_st	1.64792
hsa-miR-324-5p_st	1.64890
hsa-miR-501-5p_st	1.67560
hsa-miR-25-star_st	1.68527
hsa-miR-491-5p_st	1.69586
hsa-miR-885-5p_st	1.70522
hsa-miR-24-2-star_st	1.72839
hsa-miR-362-5p_st	1.74159
hsa-miR-100_st	1.75669
hsa-miR-421_st	1.78591
hsa-miR-34a-star_st	1.86101
hsa-miR-589-star_st	1.87267
hsa-miR-27b-star_st	1.88430
hsa-miR-29c-star_st	1.92817
hsa-miR-1281_st	2.09696
hsa-miR-138-1-star_st	2.13082
hsa-miR-212_st	2.26038
hsa-miR-769-5p_st	2.28414
hsa-miR-181c_st	2.37080
hsa-miR-184_st	2.49064
hsa-miR-455-5p_st	2.75673
hsa-miR-550-star_st	2.90365

Results from the chip were filtered to show human miRNA only and true significant changes ($p<0.05$) when compared to control.

3.8.1. miRNA validation via q-PCR

We selected 5 different miRNAs that bioinformatics site (http://www.targetscan.org) predicted may target mRNA of genes associated with iron metabolism. We attempted to validate these with q-PCR which is a more sensitive measure, to see if changes were produced. The following miRNAs were selected: hsa-let-7a; hsa-let-7d; hsa-let-7f; hsa-miR-106b; hsa-miR-92b.

There were no significant differences in any of the miRNAs when tested by q-PCR between control and quercetin treated group (Figure 3.15A). This indicates that these particular miRNAs do not possess any complementary binding site for FPN mRNA. Figure 3.15B shows how q-PCR results compare with miRNA changes measured by the Affymetrix GeneChip miRNA array method.

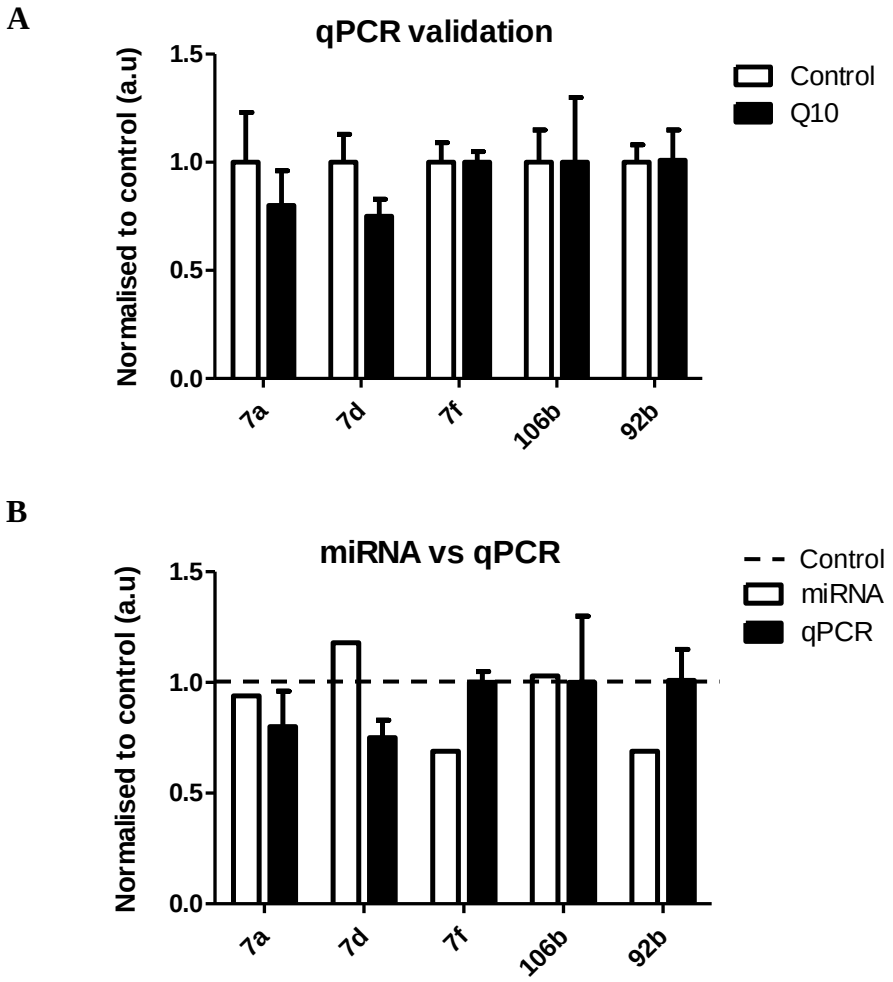


Figure 3.15 Quercetin has no effect on selected miRNAs

Caco-2 cells were treated with quercetin (10 μM) or untreated (control) for 24 hours.

(A) Changes in miRNA expression measured by q-PCR (n=8). **(B)** Changes in miRNA from array compared to q-PCR. Data normalised to corresponding control for both methods. Data analysed by t-test compared to untreated control. Data presented as mean ± SEM.

3.8.2. Transfection of 3'UTR ferroportin construct

Interactions of miRNAs with their target mRNAs may be key in gene regulation but currently very little experimental data is available. There is a large dependence on computational predictions but the lack of experimental data means the algorithms used may not be the most accurate. 3'UTR reporter constructs have been used by many researchers as a method to validate interactions between miRNAs and their potential targets. In this study we used a ferroportin 3'UTR-luciferase reporter clone to assay for endogenous miRNA activity. Pre-cloned human ferroportin 3'UTR was inserted into a viral vector which expressed an optimised luciferase reporter gene (Renilla). After 24 hours transient transfection differentiated Caco-2 cells were either left untreated or treated with quercetin (10 μM) for a further 24 hours. Results were normalised to untreated empty vector.

Transfection with 3'UTR significantly increased renilla activity (Figure 3.16; 12.24 ± 0.95; $p<0.001$) confirming transfection had correctly taken place and the 3'UTR was driving renilla reporter expression. Co-incubation of 3'UTR with 10 μM quercetin was still significantly increased compared to control (9.21 ± 1.35 $p<0.01$) but there was a reduction in renilla activity when compared to 3'UTR alone suggesting that quercetin may interfere with miRNA-3'UTR interaction to reduce reporter activity.

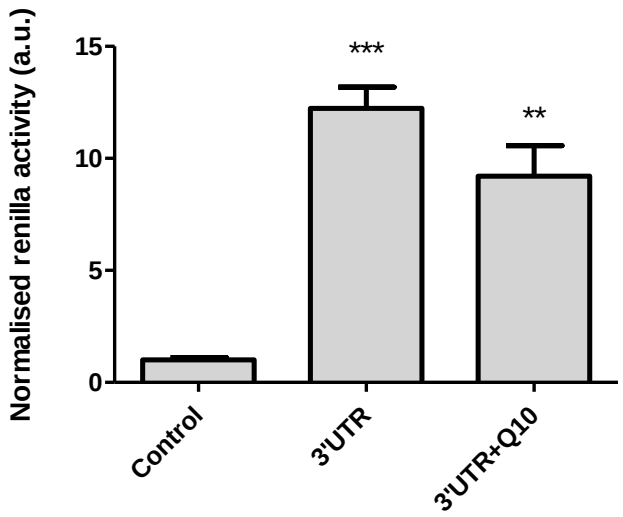


Figure 3.16 Quercetin decreased FPN 3'UTR induced renilla activity

Caco-2 cells were transfected with ferroportin (FPN) 3'UTR containing reporter plasmid for 24 hours or an empty vector (control). 3'UTR cells were treated with quercetin (10 µM) for 24 hours. Data normalised to control renilla levels. Data analysed by one-way ANOVA and Tukey's posthoc test. Data presented as mean ± SEM; n=3.

3.8.3. Quercetin significantly upregulated hsa-mR-17-3p

There is compelling evidence that hsa-miR-485-3p can regulate FPN (Sangokoya et al. 2013). Bioinformatics suggested that hsa-miR-17 might also target FPN; this miRNA was upregulated in our miRNA arrays (hsa-miR-17-5p: 1.19; hsa-miR-17-3p: 1.54) and hsa-miR-17-3p presents least four possible binding sites. Predicted potential binding sites of these miRNA to FPN 3'UTR are shown in Figure 3.17.

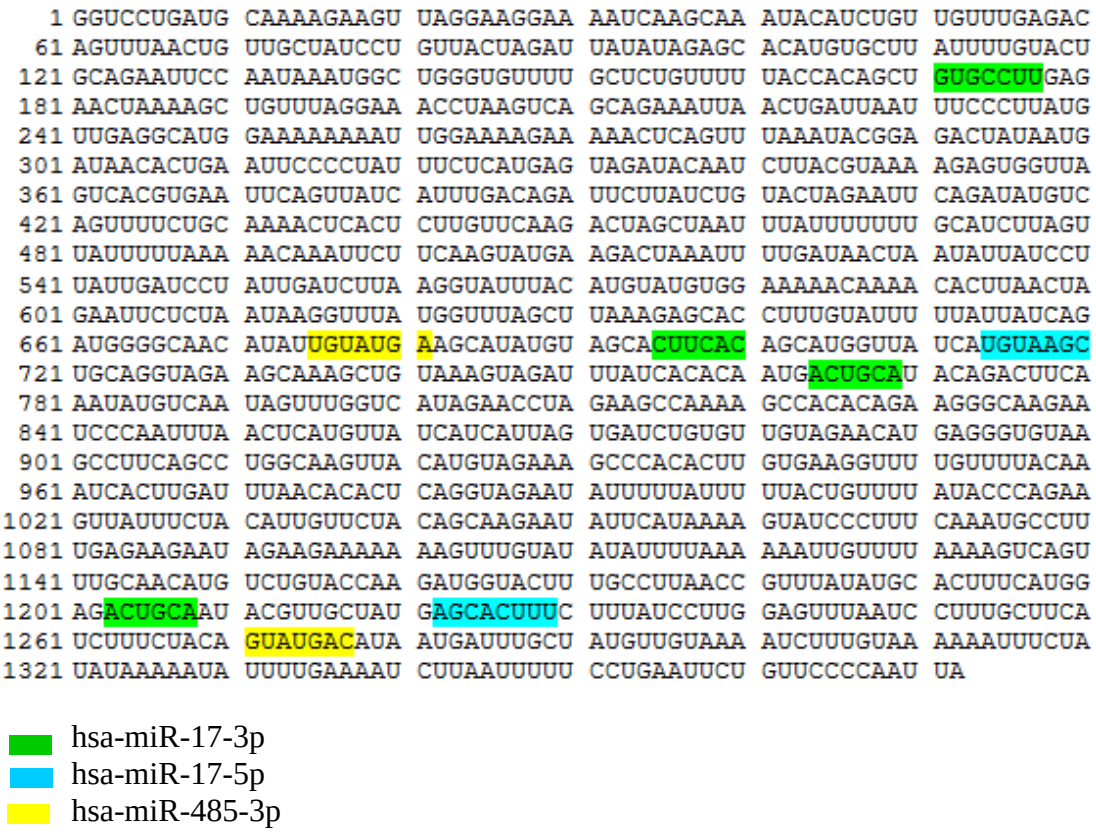


Figure 3.17 Predicted miRNA binding sites to ferroportin 3'UTR sequence

To investigate whether quercetin has a regulatory effect on these miRNAs, we isolated miRNAs from Caco-2 quercetin-treated samples and carried out q-PCR to check expression levels.

Quercetin induced over 100-fold increase in hsa-miR-17-3p expression compared to control (101.0 ± 25.1; $p<0.01$; Figure 3.18). Expression of hsa-miR-17-5p and hsa-miR-485-3p did not change significantly following quercetin treatment.

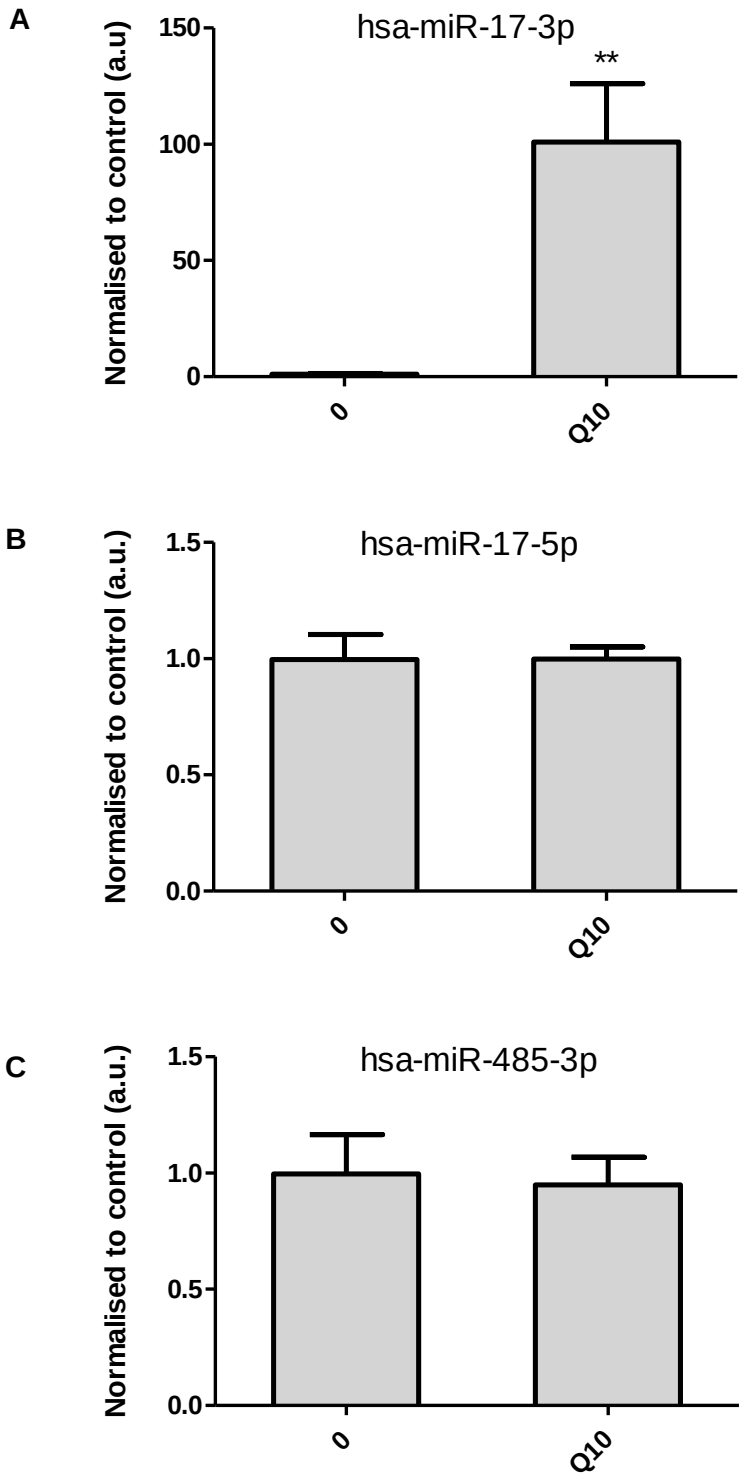


Figure 3.18 Quercetin significantly increased hsa-miR-17-3p expression

miRNA was isolated from Caco-2 cells previously treated with quercetin (10 µM) or untreated (control) for 24 hours. Changes in expression measured by q-PCR (**A**) hsa-miR-17-3p (n=5) (**B**) hsa-miR-17-5p (n=6) and (**C**) hsa-miR-485-3p (n=6). Data normalised and compared to untreated control. Data presented as mean ± SEM.

3.9. Discussion

The primary site controlling body iron levels is the small intestine which determines dietary iron absorption. Iron uptake is regulated by a number of factors and the human intestinal Caco-2 cell line provides a useful and convenient model to look in much greater detail at how individual components of the diet influence absorption.

We utilised the Caco-2 model to investigate the short-term and long-term effects of polyphenols on iron absorption. Polyphenols are generally thought to have an inhibitory effect on iron transport due to their properties as iron chelators (Afanas'ev et al. 1989). However, we found that in an acute setting (20 min), the most abundant dietary polyphenol, quercetin, significantly increased iron uptake. Similar findings were observed in previous studies using grape seed polyphenols (Kim et al. 2008). Grape seed extract and EGCG (epigallocatechin-3-gallate) acted as reducing agents favouring Fe^{2+}. Also Vlachodimitropoulou et al (2010) showed that quercetin could substitute for ascorbate as an electron donor to Dcytb, leading to reduction of iron to the absorbable ferrous form. It was also shown that quercetin could enter cells via GLUT-transporters (Vlachodimitropoulou et al. 2011). This provides a possible system for quercetin-Fe complexes to gain cellular access. Despite an increase in iron uptake, efflux following acute exposure to quercetin was decreased. This suggests the formation of intracellular quercetin-Fe complexes that are too large to be exported via FPN or direct inhibition of intracellular quercetin on the FPN transporter. Further studies are required to determine mechanisms.

Following longer-term exposure (24 hours), quercetin no longer had an effect on uptake but significant reduction of iron efflux was maintained. The data agrees with observations where single-meal studies show a significant reduction in iron absorption but individuals with long-term polyphenol intakes have no such effect (Samman et al. 2001, Reddy et al. 2000).

Polyphenols present in fruit and vegetables have long been associated with numerous health benefits (Hertog et al. 1993a) and this is often attributed to their antioxidant properties as chelators (Morel et al. 1993) and free radical scavengers (Zhao et al. 2001). However, further studies have now demonstrated that polyphenols can additionally modify gene expression. In the Caco-2 model, cDNA array analysis revealed that incubation with polyphenols epicatechin and cocoa extract altered the expression of over 40 genes (Noe et al. 2004). Changes in the expression of STAT1, MAPKK1 and ferritin genes were validated by q-PCR and western blotting. These genes, which are involved in the cellular response to oxidative stress, are in accordance with the antioxidant properties of polyphenols but also imply mechanisms of action at the molecular level. Microarray data of HUVEC cells (human umbilical vein endothelial cells) show that quercetin modifies a significant number of genes (Nicholson et al. 2008). Notas et al (2012) showed that 24 hour incubation of HepG2 cells with quercetin modified transcription and/or activity of a large number of transcription factors thereby affecting gene expression (Notas et al. 2012). Therefore to explain our results of how 24 h quercetin treatment significantly reduces iron export without affecting uptake, the gene expression of iron transporters was investigated.

Here we showed that DcytB and DMT1 mRNA were not significantly changed by quercetin treatment but there was a significant down-regulation in mRNA expression of basolateral transporter ferroportin, and its partner ferroxidase hephaestin. These observations provide a cellular mechanism that is consistent with the effects seen in the ^{55}Fe transport studies. These changes were validated at the protein level which had a mirrored response to mRNA of quercetin having no effect on DMT1 protein but a down-regulation of ferroportin protein levels. Taken together, the data creates a working model that quercetin acutely chelates iron which stimulates similar cellular responses as to an iron deficiency condition, causing enterocytes to increase iron uptake. After the initial bout, the system recovers and with time adapts so that a significant influx of iron is no longer required but cellular iron content is tightly controlled at the export level.

To further explore the hypothesis that quercetin may be inducing a state of cellular iron deficiency, we assessed the iron status of the cell by looking at ferritin and IRP2 protein as iron status biomarkers. Ferritin is a ubiquitous intracellular iron storage protein (Harrison and Arosio 1996) and the amount of ferritin stored usually reflects the amount of iron stored. Ferritin mRNA contains an IRE in its 5'UTR (Hentze et al. 1987). High or adequate levels of iron induce the degradation of IRP2 but during low levels of iron, binding of IRP2 to the IRE prevents translation of ferritin so less protein is produced. Following incubation with quercetin, ferritin protein was significantly decreased suggesting a lack of iron availability within the cell. Quercetin had no effect on IRP2 which is interesting because if quercetin was indeed causing iron deficiency we would expect an increase in IRP2. Also if the cell was iron

deficient, we would expect an increase in DMT1 (Zoller et al. 2001) and ferroportin (McKie et al. 2000) to enhance iron absorption. Therefore, although this mechanism might contribute to some of the effects seen in the acute condition, it is unlikely to be a main factor in the observed ferroportin down-regulation. Some other mechanism(s) must also be in play.

We began the search by investigating Nrf2. This transcription factor has recently received attention due to binding sites for it having been found on the macrophage ferroportin promoter (Marro et al. 2010). Further linking it to our study, it has been suggested that quercetin may regulate Nrf2 (Granado-Serrano et al. 2012). To see if this was the crucial link between the quercetin-iron-ferroportin axis, we looked at cytosolic Nrf2 protein and translocated nuclear Nrf2 protein in Caco-2 cells. The cytosol showed no change but the nuclear fraction showed a significant increase in Nrf2 protein with increasing quercetin concentration (100 µM: $p<0.001$). This is not consistent with our observations. As a transcription factor, Nrf2 binds to promoter regions to induce gene expression thus if quercetin increases nuclear Nrf2 then this should increase ferroportin expression. Interestingly, Granado-Serrano et al. (2012) showed that while 10 µM quercetin significantly increased nuclear translocation of Nrf2 in HepG2 cells, 50 µM quercetin inhibited this effect; our results in Caco-2 cells do not show this biphasic response.

Different kinases have been reported to regulate Nrf2 including ERK and PI3K and phosphorylation of Nrf2 appears to be essential for its nuclear translocation (Eggler et al. 2008, Granado-Serrano et al. 2012). As quercetin seems to increase nuclear Nrf2

but contradictorily decrease FPN expression, it could be an antioxidant effect of quercetin to modulates these kinases to affect Nfr2 rather than having a direct effect. It is likely that quercetin-induced Nrf2 changes are cell specific but in Caco-2 cells our results show it is unlikely to be the main regulatory mechanism in controlling ferroportin expression.

We then moved our attention to erythropoietin (Epo) which is known to stimulate DMT1 and FPN (Srai et al. 2010). We confirmed that Epo does indeed stimulate DMT1 and FPN and showed that quercetin is able to counteract Epo-induced stimulation on DMT1 and FPN by reverting it to basal levels (Figure 3.7). This suggested that quercetin was affecting FPN expression by an Epo-stimulatory pathway. We focused on the PI3K (phosphatidylinositol 3-Kinase) pathway as specific PI3K inhibitors have similar structural properties to quercetin ((Vlahos et al. 1994). We found that incubation with the PI3K inhibitor LY294002 inhibited the stimulatory effect of Epo on DMT1 and FPN. Interestingly, the PI3K inhibitor reduced DMT1 mRNA below control level suggesting that it might be an essential pathway required for basal DMT1 expression; the lack of effect of quercetin on DMT1 mRNA suggests that it does not operate via PI3K signalling. However in the future it would be wise to look at phospho-Akt, a downstream target of the PI3K pathway. Epo increases phosphorylation of the Akt protein (Shi et al. 2010, Ratajczak et al. 2001) and if this phosphorylation was blocked by quercetin and the PI3K inhibitor then it would confirm that they both work by the same pathway.

A recent paper by Zhang et al. (2009) showed that that there are two isoforms of ferroportin which are different in their 5'UTR promoter regions: FPN1A (+IRE) and FPN1B (-IRE). These isoforms are differentially regulated by iron. Having cloned the promoters into expression vectors, we were surprised to find that in Caco-2 cells neither iron (ferric ammonium citrate (FAC)) nor quercetin treatment had any effect on the activity of either promoter (Figure 3.10). However, at the mRNA level, quercetin did show differential effects on the two ferroportin isoforms – decreasing FPN1A but increasing the expression of FPN1B (Figure 3.14). This suggests that despite FPN1B being specific to intestinal epithelial cells, FPN1A remains the major isoform (Zhang et al 2009).

Intestinal ferroportin expression is regulated by the hypoxia-inducible factor HIF2α (Taylor et al 2012). We investigated the effect of chemical inhibitors of both HIF1α and HIF2α on ferroportin promoter activity. While neither inhibitor altered basal promoter activity, co-incubation of HIF1α inhibitor with quercetin (10 μM) significantly decreased FPN1A+IRE promoter activity ($p<0.01$) suggesting that there may be some interaction between quercetin and the HIF1α pathway in regulating ferroportin expression.

Since exposure to quercetin alone did not influence the activities of either promoter this suggests that there are no quercetin-responsive elements in the 895 (1A)/1050 (1B) base pair proximal sequence of FPN that can account for quercetin inhibition of FPN. We therefore moved to investigate the 3'UTR of FPN, focusing on microRNA (miRNA) binding sites.

miRNAs are small non-coding RNA molecules, usually of 21 to 25 nucleotides in length that negatively regulate gene expression (Wu et al. 2006). They are partially complementary to one or more mRNA molecule. miRNA research is an exciting new field which is constantly being updated. Since the early 2000, thousands of miRNAs have been discovered and the miRBase database (www.mirbase.org) hosted by the Sanger Institute, provides information on miRNA sequences and target prediction information. Recent evidence has shown that many miRNAs are activated by changes in iron status and specific miRNAs can regulate iron metabolism genes such as miR-Let-7d for DMT1(-IRE) in erythroid cells (Andolfo et al. 2010) and miR-485-3p for FPN in hepatocyte and erythroid cells (Sangokoya et al. 2013). These findings highlight the exciting possibility that miRNAs can provide an additional means of post-transcriptional iron regulation.

We carried out an Affymetrix GeneChip miRNA array to seek out miRNAs that were activated by quercetin treatment and from those chose to validate specific miRNAs that were significantly upregulated or cross-matched with the miRBase database as likely candidates involved in iron regulation. Amongst others (hsa-let-7a; hsa-let-7f; hsa-miR-106b; hsa-miR-92b. hsa-miR-17-5p; hsa-miR-17-3p), this included miR-let-7d, miR-485-3p and miR-17-3p. Our results found that miR-17-3p was significantly activated and upregulated by quercetin treatment (Figure 3.18) suggesting that quercetin modulates this miRNA as a mechanism of decreasing intestinal FPN expression. FPN 3'UTR has a number of binding sites for this miRNA and the fact that both measures of array and q-PCR show significant upregulation of miR-17-3p

shows consistency and accuracy of this data. There was no significant change in q-PCR analysis of miR-485-3p which also possesses potential binding sites on FPN 3'UTR. This could be because some of these miRNAs are cell-specific and not expressed in enterocytes; for example although miR-485-3p had a regulatory effect on FPN in hepatocyte and erythroid cells (Sangokoya et al. 2013), it was not detected in our miRNA array which used Caco-2 cells, and it was not changed when measured by q-PCR. Quercetin did not affect DMT1 mRNA and as such also did not change miR-let-7 family expression.

Upregulation of miR-17-3p by quercetin shows a novel mechanism of FPN regulation in intestinal cells. These experiments should be repeated in other cell lines to assess cell-specificity.

To confirm endogenous miRNA activity with its target 3'UTR-FPN, we transiently transfected Caco-2 cells with the ferroportin 3'UTR. 3'UTR increased reporter activity but quercetin (10 µM) caused a reduction in activity implying that quercetin is able to interfere with the miRNA-3'UTR interaction and thereby reduce reporter signalling. Overall, this suggests that quercetin can affect miRNA activity to regulate FPN. However, the diverse nature of miRNAs mean that one miRNA can have multiple mRNA targets and similarly a given target may be targeted by multiple miRNAs. Their expression and activity also varies greatly between different tissues. This makes it hard to narrow down miRNAs to a sole target or function. However, with reports of miRNA regulation of iron uptake (DMT1: miR-Let-7d) (Andolfo et al. 2010), storage (ferritin: miR-200b) (Shpyleva et al. 2011), export (ferroportin: miR-

485-3p) (Sangokoya et al. 2013), and regulation (hepcidin: miR-122) (Castoldi et al. 2011) it is quite clear that miRNAs possess important regulatory roles. If the miRNA-gene axis can be manipulated, it can be used as a therapeutic tool to treat various diseases. By using miRNA to target intracellular FPN, cellular iron status can be altered; hepcidin deficiency or resistance can be bypassed and therapeutic miRNA mimetics or inhibitors might be developed to target iron overload conditions. This research provides insight into the direct relationship between polyphenol-miRNA-FPN interactions at the intestinal iron absorption stage. Although more work is required on miRNA biology, the prospect of miRNAs as future therapeutic targets shows great promise.

4. Results II

HepG2 hepatocyte cell line

4.1. Introduction

4.1.1. Quercetin and hepcidin expression

The liver is the primary site of iron storage and therefore contains a host of iron regulatory proteins to achieve this function. The aim of this part of the study was to investigate the effects of quercetin on hepatocytes. We used HepG2 cells as an *in vitro* model of human hepatocytes. HepG2 cells are of hepatocellular carcinoma origin (Knowles et al. 1980) and are adherent, epithelial-like cells which grow as monolayers and in small aggregates. Their robust morphology and their ability to secrete a range of major plasma proteins make them a good model for studying liver metabolism.

The liver is also the site of hepcidin production. We focused on the transcriptional regulation of hepatocyte hepcidin expression as a number of putative transcription factor-binding sites have been identified in the hepcidin promoter including response elements for SMAD4 (Verga Falzacappa et al. 2008). SMAD4 is a co-SMAD protein that complexes with other receptor-regulated SMADs to serve as a transcription factor to activate the hepcidin promoter. Bone morphogenetic protein 6 (BMP6) is a key endogenous regulator of hepcidin (Andriopoulos et al. 2009). The critical role of the BMP6/SMAD pathway is highlighted in $BMP6^{-/-}$ mice (Meynard et al. 2009) and in mice with targeted disruption of SMAD4 (Wang et al. 2005) where a loss of hepcidin expression is accompanied by a huge parenchymal iron overload - a phenotype similar to that seen in haemochromatosis.

4.1.2. Iron and HepG2 cell proliferation

Iron is indispensable for DNA synthesis and deprivation can arrest cell proliferation because it is essential to many enzymes involved in cell division. One such enzyme is ribonucleotide reductase which is responsible for reducing ribonucleotides into deoxyribonucleotides for DNA synthesis. Ribonucleotide reductase contains an iron centre which is continually regenerated and is therefore sensitive to intracellular iron depletion; such depletion can inhibit ribonucleotide reduction, stop DNA synthesis and eventually cause cessation of cell growth (Nyholm et al. 1993).

Rapidly dividing cancer cells express elevated levels of the iron transporter TfR1 (transferrin receptor-1) to meet the high iron demand for rapid DNA synthesis (reviewed by Richardson et al. 2009). Iron chelating agents such as desferrioxamine have been shown to reduce the growth of tumour cells *in vitro* (Reddel et al. 1985). The chelator can cross plasma membrane to bind intracellular iron thereby limiting its bioavailability. Addition of iron to media is able to reverse the growth inhibition demonstrating that iron is the major cause of reduced proliferation (Fukuchi et al. 1994, Nyholm et al. 1993).

Quercetin is an iron chelator and some anti-proliferative effects have been demonstrated. 24-hour incubation with quercetin inhibited HeLa cell proliferation which was readily reversed by adding iron (Triantafyllou et al. 2007). However, in rat aortic smooth muscle cells, low concentrations of quercetin showed anti-proliferative effects whilst high quercetin concentrations induced the opposite effect of being pro-proliferative. (Shih et al. 2004).

We used a range of iron concentrations in the form of ferric ammonium citrate (FAC) and hemin (to represent haem) to investigate what effect they would have on HepG2 cell proliferation and whether quercetin would be able to block these effects through its chelating ability.

4.1.3. HepG2 cell viability

Studies have noted that quercetin is protective against various cancers by activating apoptotic pathways (Nguyen et al. 2004, Granado-Serrano et al. 2006). In human hepatoma HepG2 cells, it was shown that 18 hour incubation with quercetin significantly induced cell death (68% with 100 μM/L) (Granado-Serrano et al. 2006). However, this increase in cell mortality was both time- and dose-dependent. A shorter incubation time of 4 hours had no effect on cell viability and lower concentrations of quercetin (10 μM) showed evidence of pro-survival. Survival-signalling cascade in many cells involves cross-communication between PI3K-Akt and PI3K-ERKs. ERK1/2 requires continuous activation for cell survival and proliferation and it is proposed that inhibition of this by quercetin contributes to increased cell death. Low concentrations of quercetin (10 and 25 μM) however increase phosphorylation of ERK1/2 and Akt suggesting that survival of HepG2 cells is dependent on activation of cell-survival pathways most likely involving PI3K-Akt and ERK pathways (Granado-Serrano et al. 2006, Mitsui et al. 2001).

The influence of iron (FAC and haem), hepcidin and quercetin on HepG2 viability was measured using the colorimetric MTS assay. As the PI3K pathway is strongly

implicated in the regulation and survival of cells, we also examined the effect of a PI3K inhibitor on iron-induced viability.

4.2. Results

4.2.1. Effect of quercetin on gene expression of iron-related proteins

We investigated the effect of quercetin on: DMT1, FPN, BMP6, SMAD4 and hepcidin. HepG2 cells were grown on 12-well plates for 24 hours and were treated with quercetin (0, 0.1, 1, 10 and 100 µM) for a further 24 hours. This was followed by RNA isolation and quantitative-PCR to measure changes in mRNA expression.

Unlike in Caco-2, quercetin induced a significant and dose-dependent decrease in DMT1 expression ($p<0.01$; Figure 4.1). FPN, similar to Caco-2 was also significantly down-regulated. There was no significant effect of quercetin on BMP6 or SMAD4 mRNA expression. However, hepcidin levels were increased significantly at 100 µM (3.29 ± 0.67; $p<0.001$).

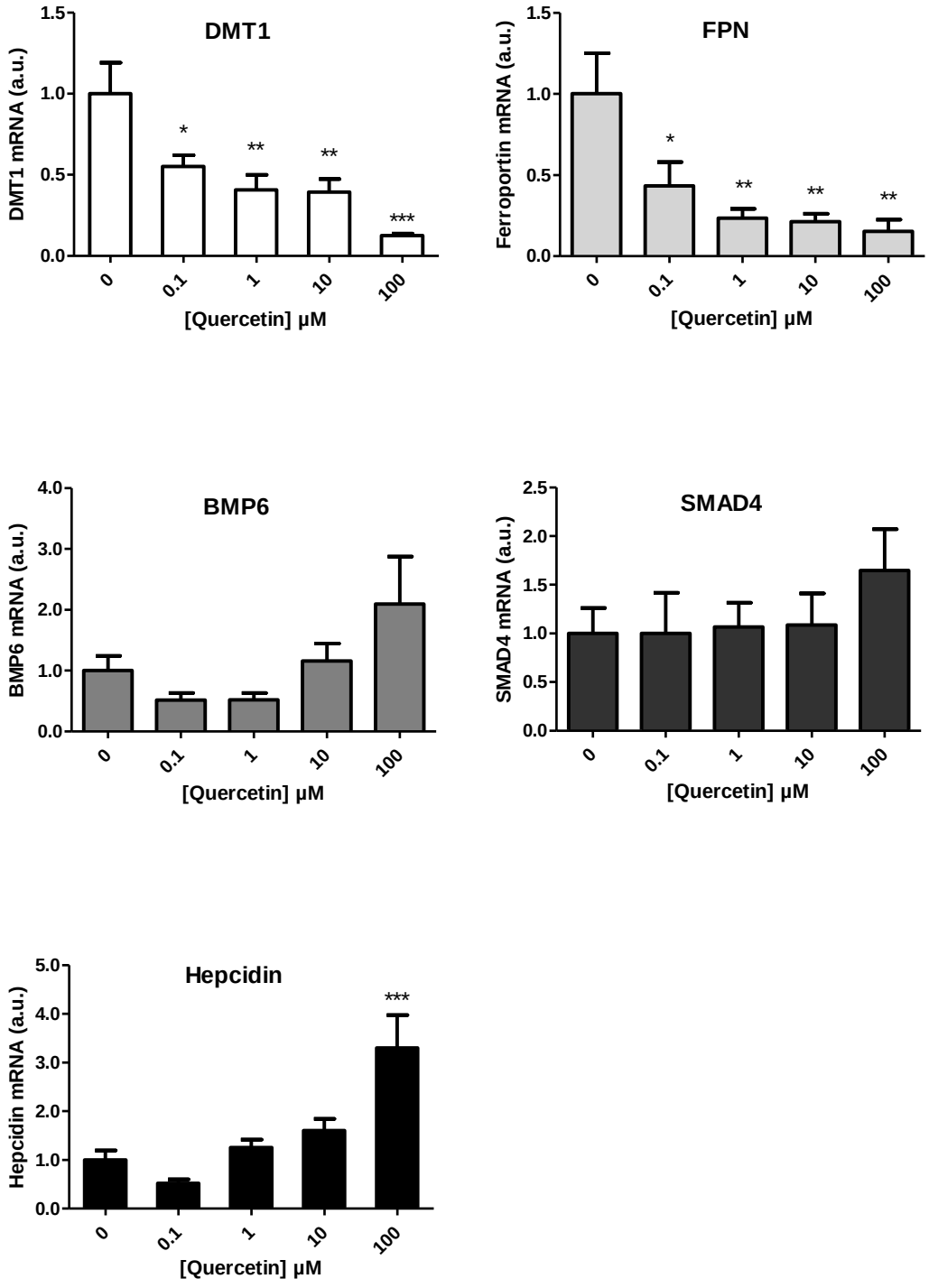


Figure 4.1 Quercetin decreased DMT1 and FPN but increased hepcidin expression

HepG2 cells were treated with quercetin (0, 0.1, 1, 10, 100 µM) for 24 hours. Changes in mRNA expression were measured by q-PCR. Data normalised to 18S. Data analysis by one-way ANOVA and Dunnett's posthoc compared to untreated control (0 µM). Significance:*p<0.05 **p<0.01 ***p<0.001. N = 8; Data presented as mean ± SEM.

4.2.2. Dose-response of FAC, haem, hepcidin and quercetin on cell proliferation

HepG2 cells were grown in 96-well plates for 24 hours. Media was replaced and treatment added to cells, along with the BrdU label (Calbiochem, Cat No HTS01) for further 24 hour incubation. Cells were treated with FAC, haem (0, 10, 50, 100 μM), hepcidin (0, 0.01, 0.1, 1 μM) or quercetin (0, 1, 10, 100 μM). Thereafter the manufacturer's protocol was followed to measure proliferation.

FAC had no significant effect on HepG2 cell proliferation (Figure 4.2). Haem, however, increased proliferation, significantly at 50 and 100 μM ($p<0.05$). Hepcidin had no effect. Quercetin at 10 μM increased proliferation (1.62 ± 0.1; $p<0.05$) but at 100 μM caused a slight reduction (0.79 ± 0.1).

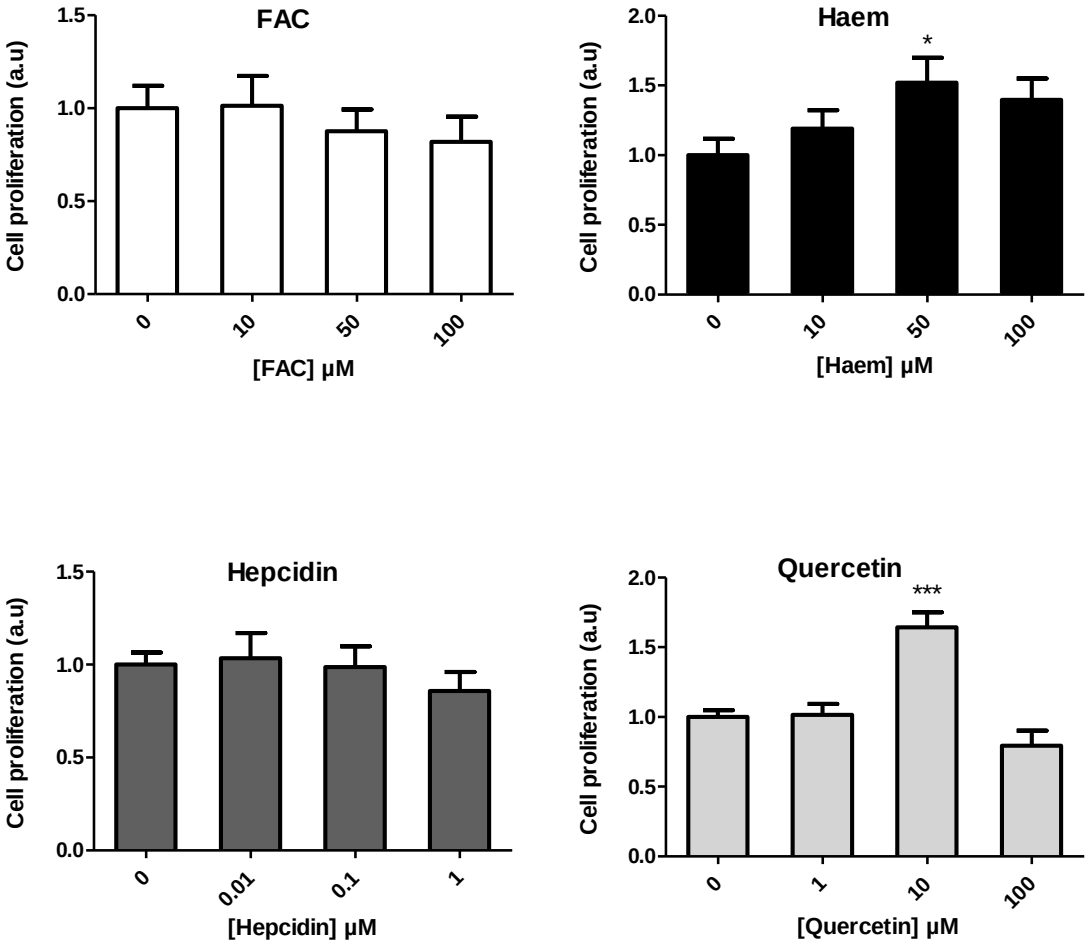


Figure 4.2 Dose-responses of FAC, haem, hepcidin and quercetin on HepG2 proliferation

HepG2 cells were treated with FAC, haem (0, 10, 50, 100 µM), hepcidin (0, 0.01, 0.1, 1 µM) or quercetin (0, 1, 10, 100 µM) and BrdU for 24 hours. Proliferation was measured by fluorescent BrdU assay. Data normalised to positive control. Data analysis by one-way ANOVA and Dunnett's posthoc.

4.2.3. Quercetin increased FAC-proliferation but decreased haem-proliferation

To look at the effect of quercetin on FAC/haem-induced proliferation, increasing concentrations of quercetin (1, 10, 100 µM) or hepcidin (0.01, 0.1, 1 µM) were added to 30 µM FAC or haem (a set concentration of 30 µM gives consistency between the two forms of iron used).

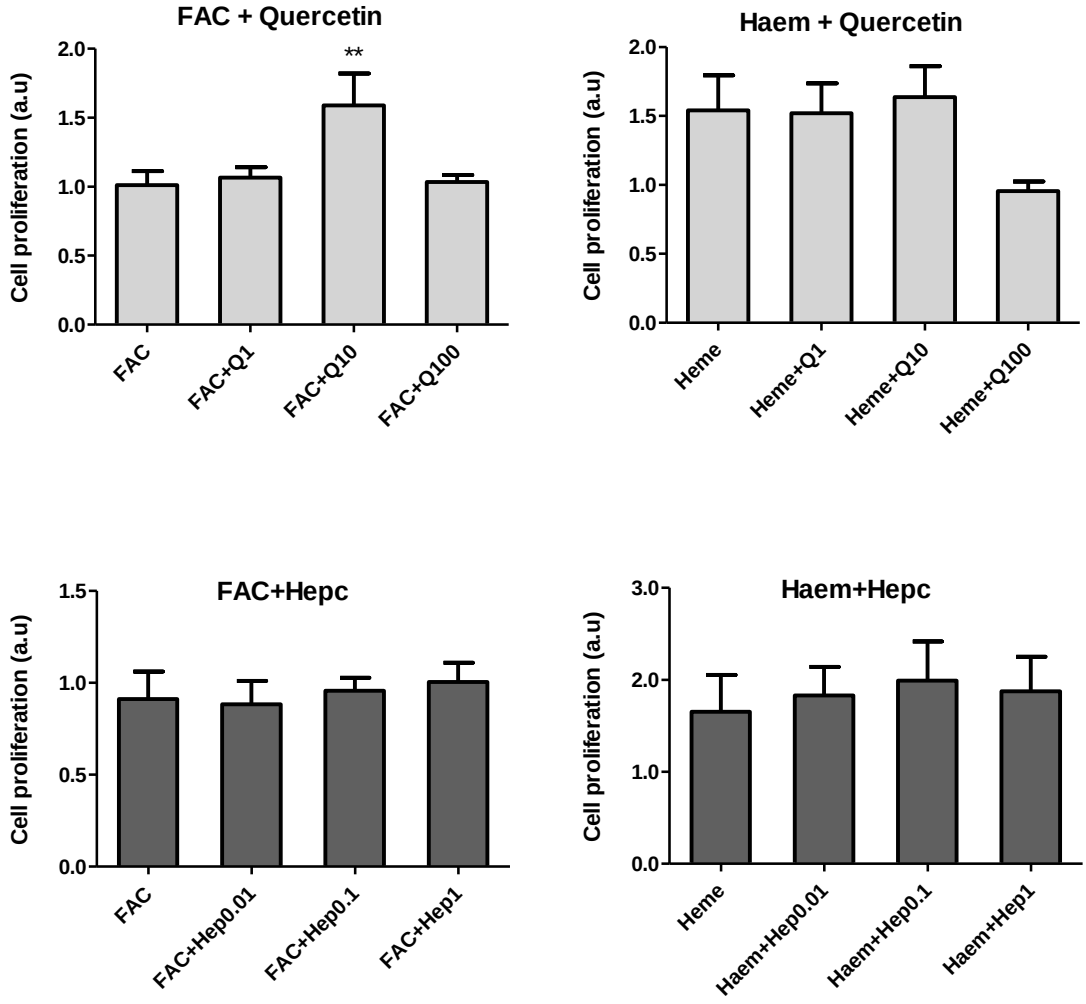


Figure 4.3 Quercetin increased proliferation with FAC

HepG2 cells were treated for 24 hours with FAC or haem combined with quercetin (1, 10, 100 µM) or hepcidin (0.01, 0.1, 1 µM) and BrdU. Data normalised to positive control. Data analysis by one-way ANOVA and Dunnett's posthoc compared to FAC or haem alone. Significance: **p<0.01. N=9; Data presented as mean ± SEM.

Hepcidin, which is proposed to increase cell iron content by blocking ferroportin, did not affect cell proliferation in response to either FAC or haem challenge (Figure 4.3). Quercetin at 100 µM caused an inhibition of haem-induced proliferation by returning proliferation to basal levels (0.96 ± 0.07). Quercetin at 1 or 100 µM did not affect FAC response but 10 µM still significantly increased proliferation even in the presence 30 µM FAC (1.59 ± 0.02; $p<0.01$; Figure 4.3).

4.2.4. PI3K inhibitor does not affect FAC-/haem-induced proliferation

PI3 kinase (PI3K) is a molecule central to cellular growth, proliferation and survival. Work has shown that in some situations quercetin is capable of inhibiting PI3K activity (Hwang et al. 2009). In order to establish whether quercetin's effects on cell proliferation were by inhibition of PI3K, a PI3K inhibitor (LY294002) was added to cells alongside FAC or haem. The PI3K inhibitor (20µM) was initially added alone to determine any baseline effects on cell proliferation, however, no significant difference was noted (Figure 4.4).

PI3K inhibitor had no significant effect on either FAC or haem-induced proliferation as compared to when treated alone (Figure 4.4).

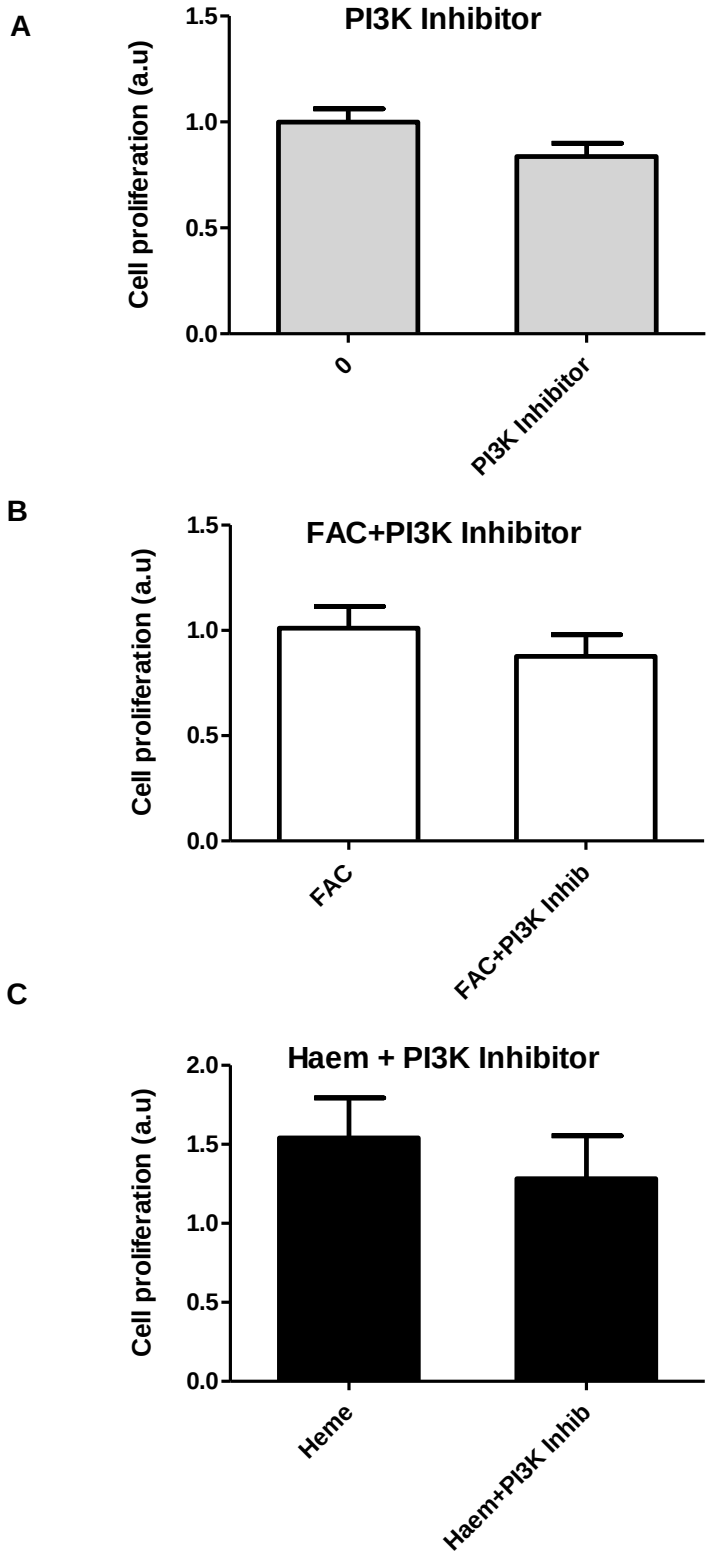


Figure 4.4 PI3K inhibitor has no significant effect on cell proliferation

Cells treated for 24 hours with **(A)** PI3K inhibitor (20 µM) or in combination with **(B)** FAC or **(C)** haem (30 µM) and BrdU label. Data normalised to positive control. Data analysis by one-way ANOVA and Dunnett's posthoc test. N=5; Data presented as mean ± SEM.

4.2.5. Dose-response of FAC, haem, hepcidin and quercetin on cell viability

HepG2 cells were grown in 24-well plates for 24 hours. Media was replaced and treatment added to cells for another 24 hour incubation period. Cells were treated with FAC, haem (0, 10, 50, 100 μM), hepcidin (0, 0.01, 0.1, 1 μM) or quercetin (0, 1, 10 μM). The manufacturer's protocol was followed to read absorbance at 485 nm as a measure of viability.

FAC significantly decreased cell viability by approximately 50% at all concentrations ($p<0.05$; Figure 4.5). Haem, hepcidin and quercetin did not significantly alter cell viability. Quercetin at a concentration of 100 μM was not included in the analysis as its solution colour interfered with absorbance readings.

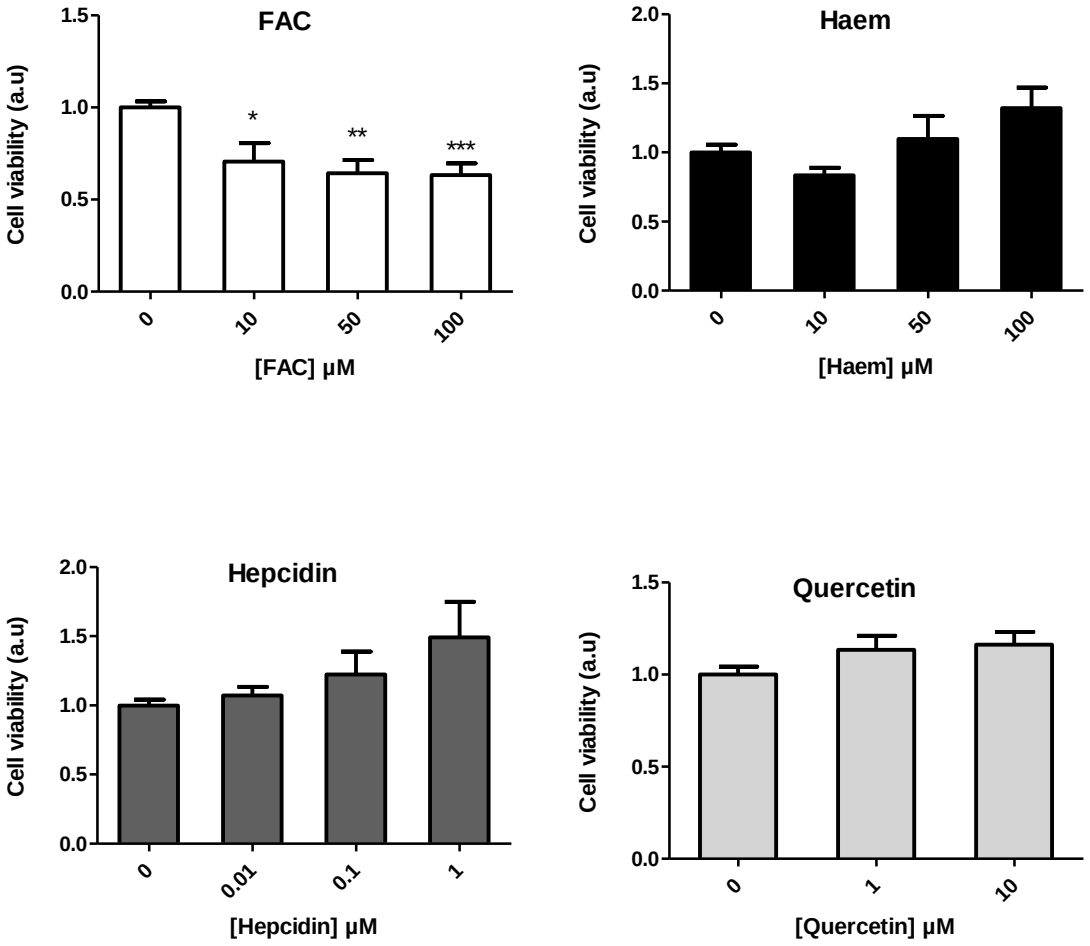


Figure 4.5 Dose-responses of FAC, haem, hepcidin and quercetin on HepG2 viability

HepG2 cells were treated with FAC, haem (0, 10, 50, 100 µM), hepcidin (0, 0.01, 0.1, 1 µM) or quercetin (0, 1, 10 µM) for 24 hours. Viability measured by MTS assay. Data analysis by one-way ANOVA and Dunnett's posthoc compared to untreated control (0 µM). Significance:*$p<0.05$ **$p<0.01$ ***$p<0.001$. N = 9; Data presented as mean ± SEM.

4.2.6. Quercetin does not affect viability with FAC or haem

To study the effects of quercetin and hepcidin on FAC/haem-induced viability, increasing concentrations of quercetin (1, 10 µM) and hepcidin (0.01, 0.1, 1 µM) were added to 30 µM FAC or haem. FAC did not reduce cell viability when added with quercetin. Quercetin had no effect on haem. Hepcidin had no effect on FAC or haem-induced cell viability.

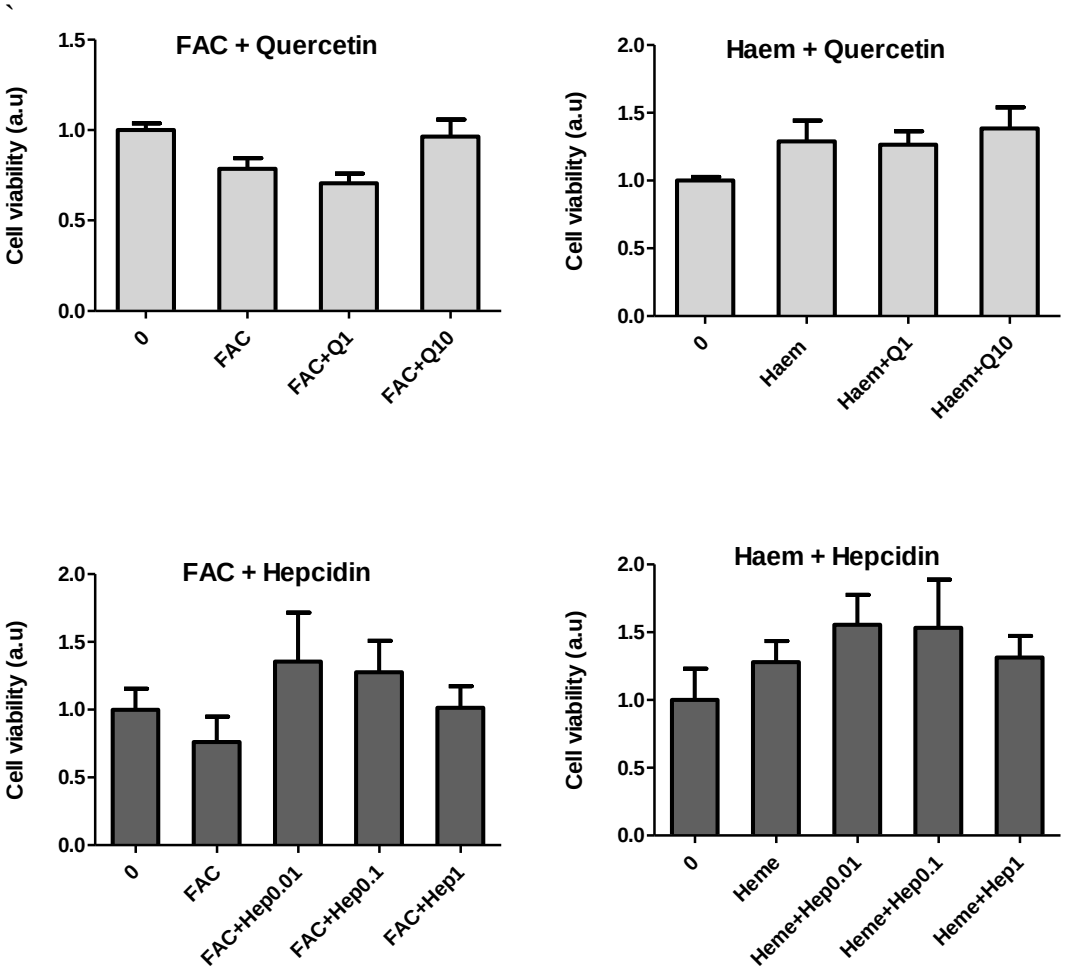


Figure 4.6 Quercetin has no effect on viability with FAC or haem

HepG2 cells were treated for 24 hours with FAC or haem combined with quercetin (1, 10 µM) or hepcidin (0.01, 0.1, 1 µM). Data analysed by one-way ANOVA and Dunnett's posthoc compared to FAC or haem alone. N=6; Data presented as mean ± SEM.

4.2.7. PI3K inhibitor does not affect FAC-/haem-induced proliferation

We used a PI3K inhibitor (LY294002 20 μM) to determine what effect it had on iron-induced viability. This was added in combination with 30 μM FAC or haem to cells. The PI3K inhibitor was initially added alone to determine baseline effects on cell viability, however, no significant difference was noted (Figure 4.7 top panel).

PI3K inhibitor had no significant effect on either FAC or haem induced viability when compared to control (Figure 4.7).

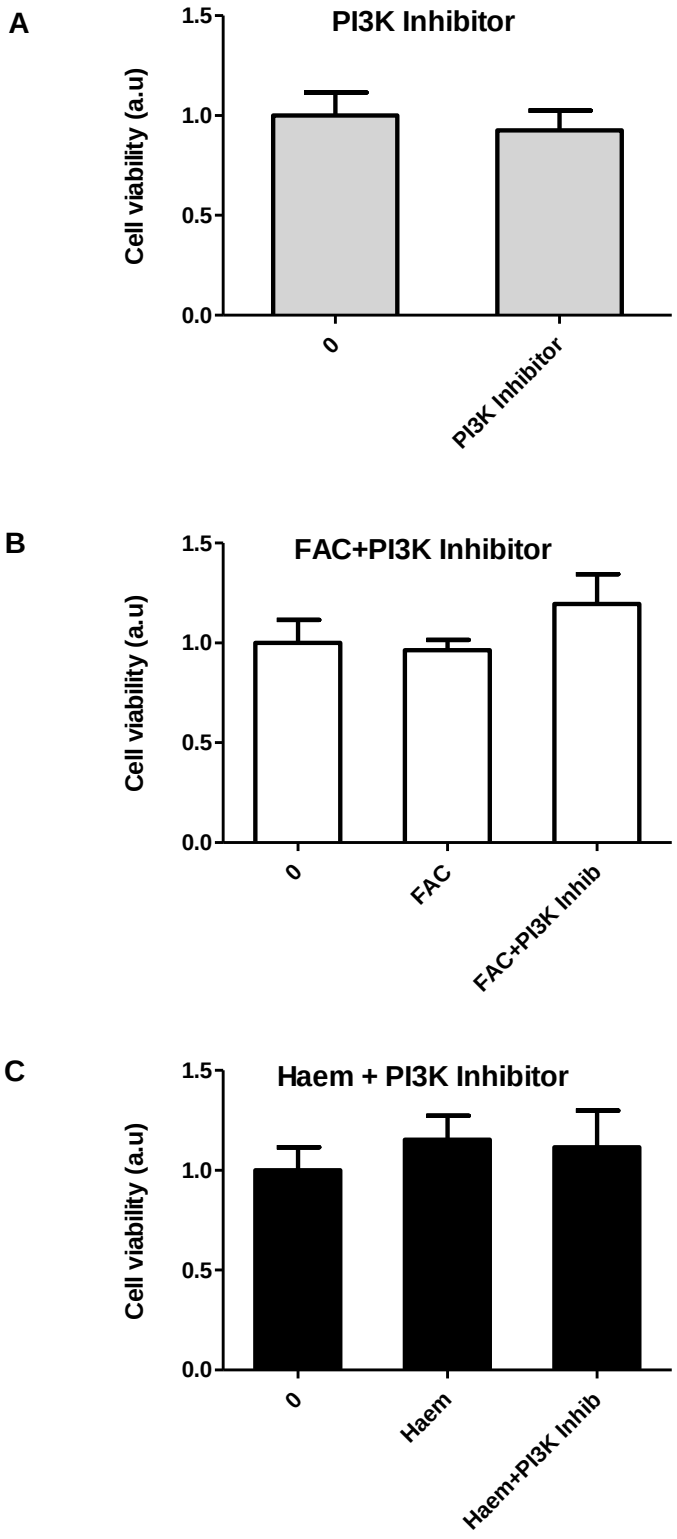


Figure 4.7 PI3K inhibitor has no significant effect on cell viability

Cells treated for 24 hours with **(A)** PI3K inhibitor+ (20 µM) or in combination with **(B)** FAC or **(C)** haem (30 µM). Data analysed by one-way ANOVA and Dunnett's posthoc compared to untreated control (0). N=6; Data presented as mean ± SEM.

4.3. Discussion

4.3.1. Iron metabolism gene expression

The liver is a very important organ for iron metabolism. Not only is it the main site of iron storage but it also produces hepcidin, often regarded as the master regulator of iron homeostasis (Drakesmith and Prentice 2012). Hepatic hepcidin synthesis is stimulated by high levels of systemic iron (Pigeon et al. 2001). Binding of hepcidin to FPN causes FPN degradation which prevents iron export to circulation, creating a negative feedback loop to inhibit further hepcidin release (Nemeth et al. 2004a).

In this study we investigated the effects of quercetin on hepcidin expression, and on levels of the iron transporters DMT1 and FPN. Quercetin is a potent iron chelator (Afanas'ev et al. 1989) and data from the earlier Caco-2 cells studies suggest that quercetin treatment reduces intracellular iron concentration. We therefore hypothesized that quercetin should decrease hepcidin and FPN but increase DMT1 expression. We found that increasing concentrations of quercetin caused a significant dose-dependent inhibition of ferroportin gene expression (Figure 4.1). However, quercetin treatment induced a dose-dependent increase in hepcidin mRNA. It is tempting to speculate that this might be part of an autocrine loop linking hepcidin and FPN expression similar to the one observed in human monocytes (Theurl et al. 2008); however, it is possible that these are separate quercetin-mediated transcriptional events.

Interestingly DMT1 expression was significantly down-regulated with as little as 0.1 µM quercetin ($p<0.05$; Figure 4.1). This is in contrast to the Caco-2 cells studies where DMT1 remained unaltered by even 100 µM quercetin. This is likely due to differential expression of DMT1 isoforms (Hubert and Hentze 2002). In addition, the decrease in DMT1 argues against quercetin-induced cellular iron deficiency as the major mechanism controlling iron metabolism gene expression in HepG2 cells. Measurement of ferritin stores and IRP activity would be appropriate to determine the extent of changes in cellular iron status following quercetin treatment.

In an attempt to explain how quercetin was inducing hepcidin expression we looked at some intermediates in the hepcidin signaling cascade: BMP6 and SMAD4. Binding of the iron-regulated BMP6 to its receptors propagates a signalling cascade that leads to phosphorylation of SMAD effector proteins (1/5/8). Once phosphorylated, they complex with the common mediator SMAD4 and translocate to the nucleus where they modulate transcription of hepcidin (Meynard et al. 2009). The importance of this pathway for hepcidin expression has been highlighted in BMP6-deficient mice (Meynard et al. 2009) and mice with targeted disruption of SMAD4 (Wang et al. 2005).

Both BMP6 and SMAD4 levels were higher in the Q100 treatment group but did not reach statistical significance. This again argues against an iron-mediated response at the mRNA level. However, future studies should also focus on BMP6 protein levels, cellular distribution of SMAD4 (nuclear and cytosolic) and the phosphorylation of the

receptor-activated SMADs 1,5,8 to determine whether these factors are influenced by quercetin in hepatocytes.

4.3.2. Effects of quercetin on cell proliferation and cell viability

As iron is essential for cell growth, we tested the effect of iron presented in two different forms: FAC and haem. FAC did not affect proliferation (0 - 100 μM) but haem (50 μM) caused a significant increase of HepG2 cell proliferation (Figure 4.2). This suggests that the form of iron presented to cells is an important determinant of proliferation. This is consistent with the observation in erythroid cells where proliferation was promoted by haem but not FAC (Leimberg et al. 2008). A study suggested that the function of iron within a cell is dependent on the form in which it is initially presented (Alcantara et al. 1991). They showed that transferrin-bound iron was channelled to regulatory functions (indicated by increased PKC-β mRNA) but soluble iron in the form of FAC was preferentially channelled to storage sites. Therefore, haem could be acting in a similar manner to iron-transferrin and be channelled into regulatory functions and this is how it contributes to increased cell proliferation whereas FAC is used for storage. To study this proposed mechanism, PKC-β mRNA should be measured to see if haem is stimulating this similar to transferrin (or some other marker of cell function) and ferritin measured to confirm whether FAC is being channelled to storage sites which would explain its lack of effect on proliferation.

Quercetin significantly increased proliferation at 10 μM (1.62 ± 0.1) but was reduced at 100 μM (0.79 ± 0.1) demonstrating both pro- and anti-proliferative effects

depending on the concentration used (Figure 4.3). These findings are supported by the work of (Shih et al. 2004) who used rat aortic smooth muscle cells to show that quercetin can have both proliferative and anti-proliferative effects, depending on the concentration used. Interestingly, 100 μM quercetin returned haem-induced proliferation to basal levels showing that quercetin can decrease proliferation as well. This is consistent with an iron chelation effect.

Studieshave demonstrated that quercetin can directly inhibit PI3K to affect proliferation (Hwang et al. 2009, Weber 2005) therefore we used a PI3K inhibitor to see if it would mimic the responses of quercetin if quercetin was truly using the PI3K pathway. However the PI3K inhibitor on its own or with FAC/haem had no effect on proliferation or cell viability indicating that quercetin is not using this pathway to affect these functions in hepatocytes.

Hepcidin did not significantly alter cell proliferation, either when given alone or in combination with FAC or haem. Hepcidin is thought to block FPN and increase cellular iron (Nemeth et al. 2004b); however, further studies will be required to determine whether cellular iron levels altered significantly in the presence of hepcidin in our studies.

In contrast to proliferation, FAC appears to have the more significant role on cell viability, illustrated by the significant dose-dependent decrease in cell viability with increased concentrations of FAC (Figure 4.5). This effect can be attributed to increased production of reactive oxygen species resulting in increased iron-dependent

cellular damage similar to that observed in other cell lines (Hoepken et al. 2004). However this effect was pacified with the addition of quercetin. Although quercetin on its own did not affect viability, it was able to bring FAC-viability back to basal levels. This is likely an antioxidant effect of quercetin. Along with its iron chelation ability, quercetin has been proposed to activate the MAPK pathway leading to expression of survival (c-Fos, c-Jun) and defensive genes (phase II detoxifying enzymes; glutathione S-transferase, quinone reductase) (Schroeter et al. 2001, Kong et al. 2000) reviewed by Williams et al. 2004). Possible activation of these cyto-protective genes suggests a quercetin-mediated inhibition of oxidative stress-induced apoptosis.

5. Results III

Thp1 macrophage cell line

5.1. Introduction

Macrophages are responsible for degrading haemoglobin from senescent erythrocytes which allows haem iron to be extracted and released back in tothe circulation for further use. The system demonstrates an efficient iron recycling system to maximise iron utilisation and minimise iron loss.

Having looked at iron absorption and storage, we next wanted to investigate the effects of quercetin on macrophages to see how it would compare with other iron compartments. We used Thp1 cells as an *in vitro* model of human macrophages. Thp1 cells originate from an acute monocytic leukemia cell line (Tsuchiya et al. 1980). In culture, these monocytes float but with the addition of phorbol-12-myristate-13-acetate, commonly known as PMA, the monocytes differentiate into macrophages and adhere to cell culture dishes. They have a doubling rate of 2 - 3 days which makes them convenient for laboratory use.

Initially we investigated the effect of quercetin on DMT1 and FPN. TRPML (transient receptor potential mucolipin subtype) channels found in intracellular organelles, particularly endosomes and lysosomes have been shown to function as iron-permeable channels in HeLa and HEK cell lines (Dong et al. 2008). The role of TRPML channels in iron metabolism in human macrophages has not yet been investigated. Thus we also looked at TRPML1 and TRPML2 (TRPML3 is not permeable to iron) to see how they would be affected by quercetin and iron in human Thp1 macrophages.

5.2. Effect of quercetin on macrophage iron transporters

Thp1 cells were grown on 12-well plates for 24 hours and were treated with quercetin (0, 1, 10 and 100 µM) for a further 24 hours. This was followed by RNA isolation and quantitative-PCR to measure changes in mRNA expression.

We found that 1 – 10 µM quercetin had no effect on DMT1, TRPML1 or TRPML2 expression. However 100 µM quercetin caused a significant down-regulation of all these transporters (Figure 5.1).

Quercetin has an opposite effect on FPN in Thp1 cells than it did in Caco-2 and HepG2 cells as quercetin caused a significant dose-dependent increase of FPN expression at all concentrations ($p<0.05$; Figure 5.1).

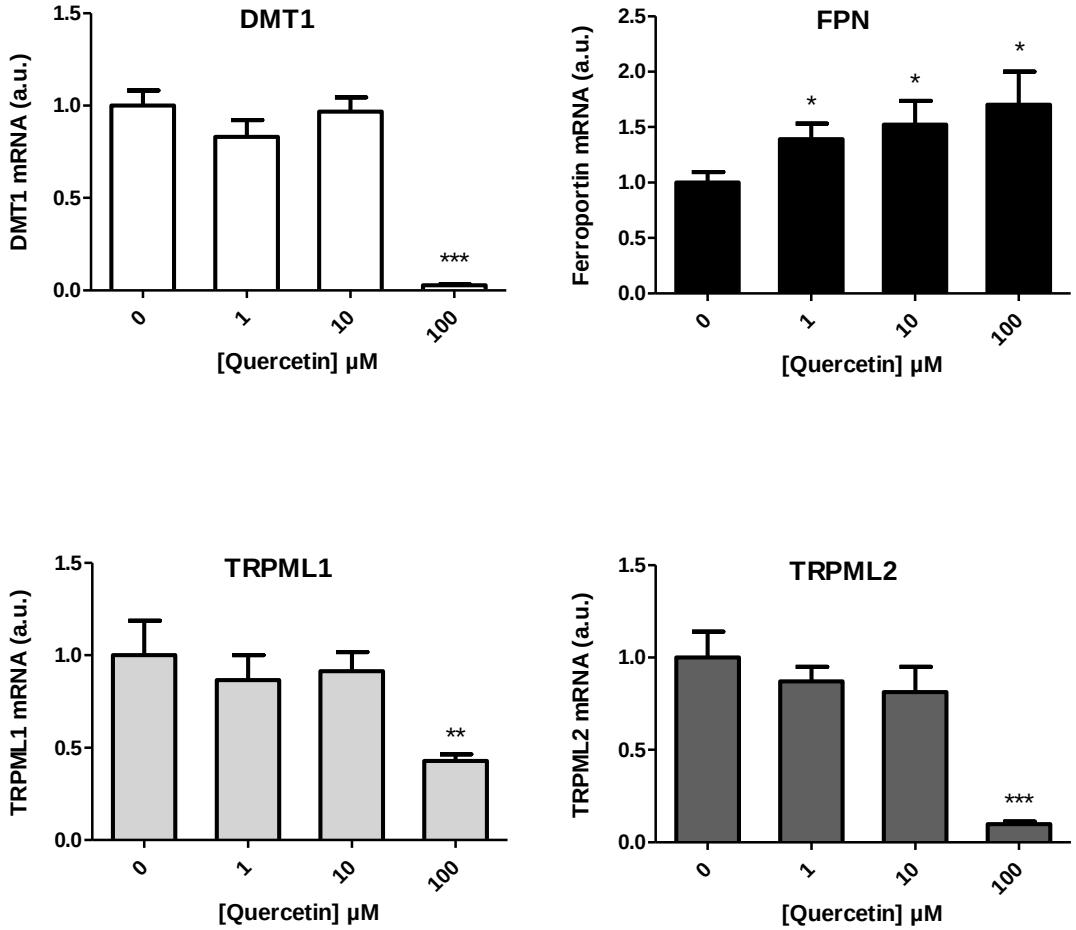


Figure 5.1 Quercetin increased FPN mRNA expression

Thp1 cells were treated with quercetin (0, 1, 10, 100 µM) for 24 hours. Changes in mRNA expression were measured by q-PCR. Data normalised to housekeeping gene 18S. Data analysed by one-way ANOVA and Dunnett's posthoc compared to untreated control (0). Significance:*p<0.05 **p<0.01 ***p<0.001. N=16 (DMT1); 18 (FPN); and 10 (TRPML1/2). Data presented as mean ± SEM.

5.3. Effect of iron on macrophage iron transporters

As macrophages are responsible for recycling iron, it was of interest to investigate whether altered cellular iron concentrations affected the gene expression of these putative iron transporters. Cells were treated with 50 μM hemin for 24 hours or left untreated. We specifically measured DMT1, Nramp1, TRPML1, TRPML2 and FPN by q-PCR.

DMT1 gene expression did not change with increased iron content (0.97 ± 0.37; (Figure 5.2). However, all other iron transporters (Nramp1, TRPML1, TRPML2, FPN) had significantly reduced mRNA expression with hemin treatment ($p<0.05$; Figure 5.2).

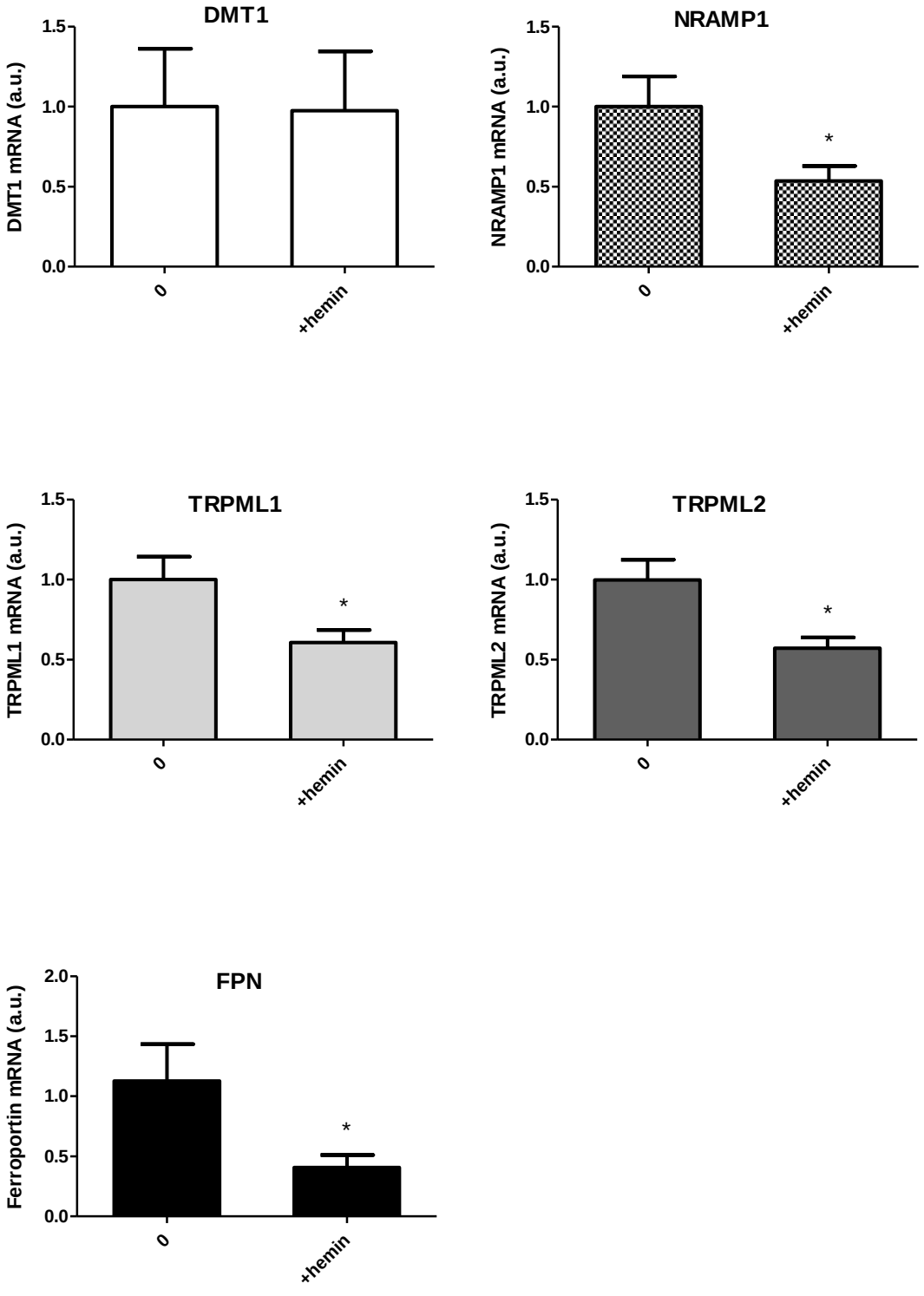


Figure 5.2 Hemin significantly reduces gene expression of most iron transporters

Thp1 cells were treated with hemin (50 µM) for 24 hours and mRNA expression measured by q-PCR. Data normalised to housekeeping 18S. Data analysed by t-test compared to untreated control (0). Significance:*$p<0.05$. N=7. Data presented as mean ± SEM.

5.4. Ferroportin 5'UTR promoter

Both variants of the FPN prompter (FPN1A and FPN1B) were transiently transfected into Thp1 cells. We measured transfection efficiency to be sure that the promoter had been correctly inserted and was driving firefly luciferase expression. FPN1A caused over a 7-fold increase in luciferase activity compared to the empty, promoter-less control vector ($p<0.01$; Figure 5.3). FPN1B caused a significant 5.5-fold increase compared to the empty pGL4 vector ($p<0.0001$).

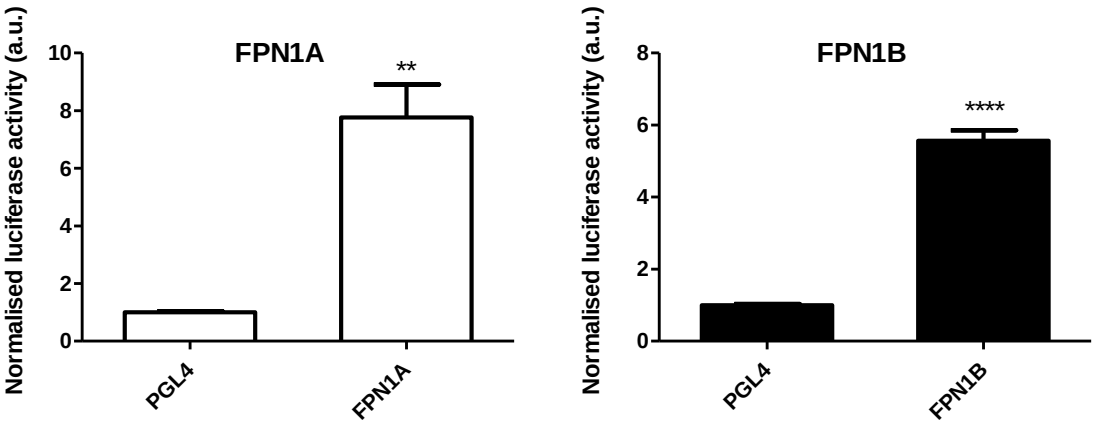


Figure 5.3 Transfection efficiencies of FPN1A/1B as compared to empty PGL4 vector

Thp1cells were incubated for 24 hours after transfection and harvested for the Luciferase assay. Data analysed by t-test compared to empty pGL4 vector. Significance: **$p<0.01$ **** $p<0.0001$; N=3; Data presented as mean ± SEM.

5.4.1. Effect of quercetin and iron on FPN1A/FPN1B promoters

To examine the responses of both promoter constructs to iron loading, Thp1 cells were transiently transfected with the FPN1A or FPN1B plasmid for 24 hours. Media was then replaced and cells were treated with quercetin (10 µM), hemin (50 µM) or quercetin and hemin combined for 24 hours.

Quercetin caused significant induction of both FPN promoter transcripts (FPN1A 1.56 ± 0.11 $p<0.05$; FPN1B 1.36 ± 0.08 $p<0.01$) (Figure 5.4). Iron loading with the addition of hemin alone or together with quercetin had no effect on promoter activities.

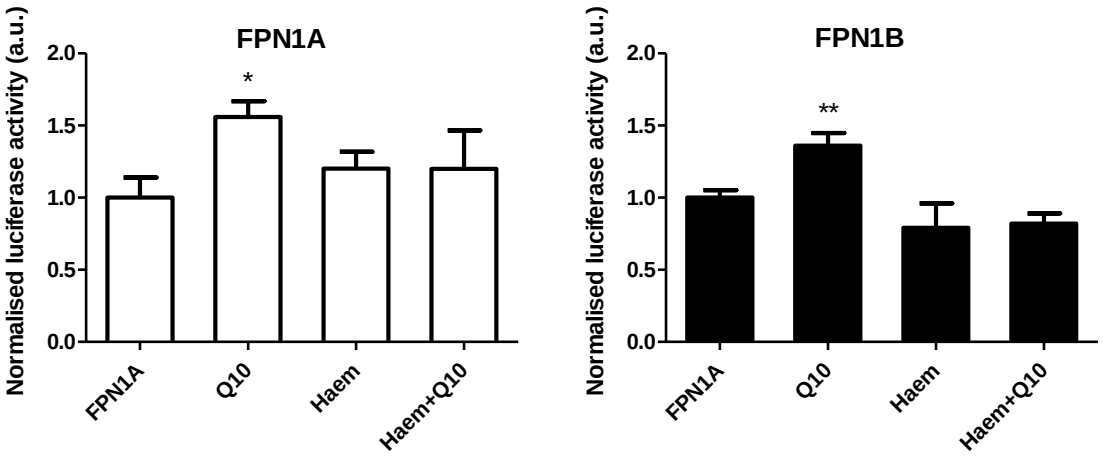


Figure 5.4 Quercetin significantly increased FPN1A/1B promoter activities

Thp1 cells were incubated 24 hours after transfection and treated with quercetin (10 µM) and/or hemin (50 µM) for further 24 hours. Data was normalised to untreated control (FPN1A or FPN1B alone). Data analysed by one-way ANOVA and Dunnett's post-hoc test. Significance: *$p<0.05$ ** $p<0.01$; N=6. Data presented as mean ± SEM.

5.4.2. Effect of Epo on FPN1A/1B promoters

As Epo has been shown to stimulate the activity of FPN1B but not FPN1A in Caco-2 cell, we investigated whether this differential stimulatory effect of Epo would also occur in human macrophages. Thp1 cells were treated with recombinant erythropoietin (Epo) (1 U/ml) and in combination with quercetin (10 µM) for 24 hours.

Epo did not induce significant activity of either promoter, when treated alone or with quercetin (Figure 5.5).

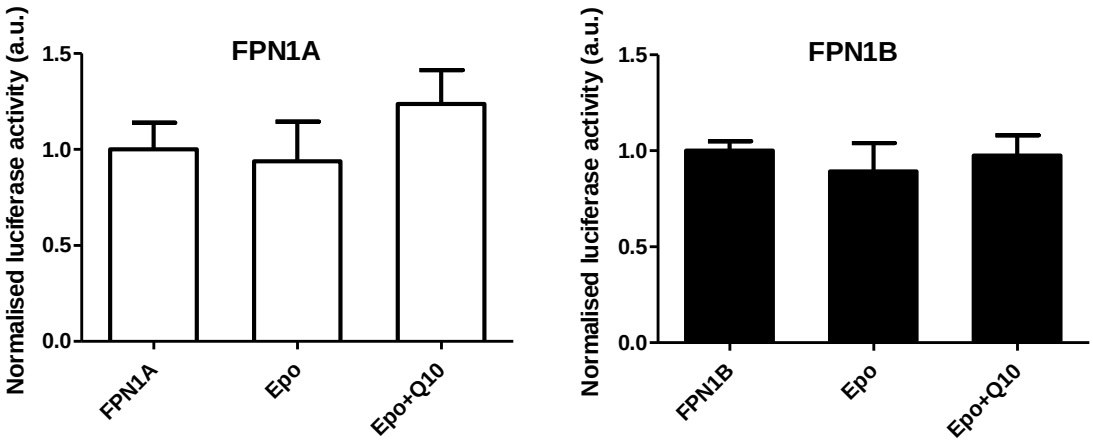


Figure 5.5 Epo has no effect on ferroportin promoters

Thp1cells were incubated 24 hours after transfection and treated with, Epo (1U/ml) or together with quercetin (10 µM) for another 24 hours. Data was normalised to untreated control (either FPN1A or FPN1B alone). Data analysed by one-way ANOVA and Dunnett's post-hoc test. N=6; Data presented as mean ± SEM.

5.4.3. Effect of HIF1α and HIF2α inhibitors on FPN1A/1B promoters

We examined the effect of HIFs on ferroportin promoters by using HIF1α (20 μM) inhibitor and HIF2α (10 μM) inhibitor. The HIF inhibitors were also co-incubated with quercetin (10 μM).

We found that neither HIF1α inhibitor nor HIF2α inhibitor had any significant effect on the FPN promoters (Figure 5.6). Co-incubation with quercetin caused a slight reduction of FPN1A promoter activity but this was not statistically significant.

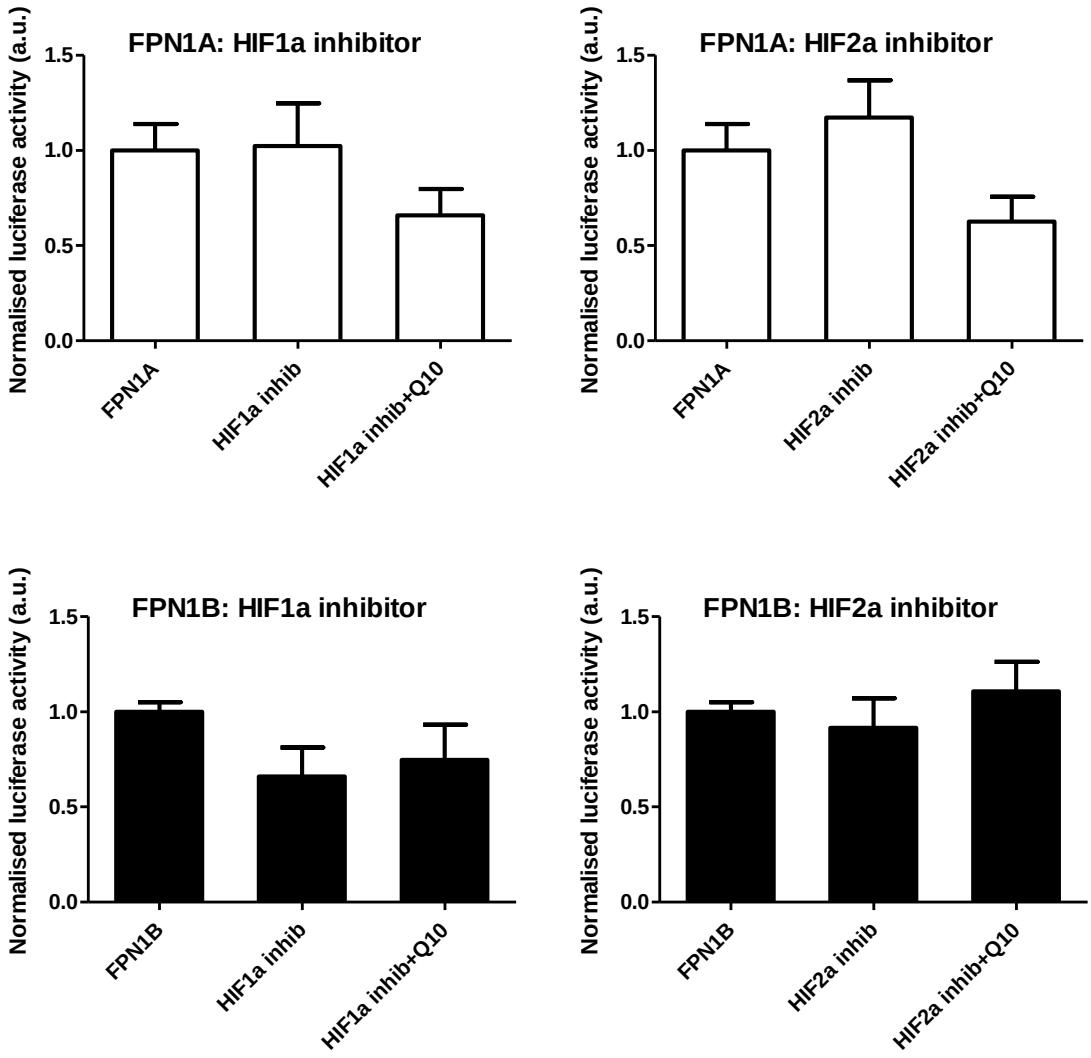


Figure 5.6 HIF1α and HIF2α inhibitors have no effect on FPN1A/FPN1B promoter activities

Thp1 cells were treated with HIF1α inhibitor (20 μM) or HIF2α inhibitor (10 μM) either alone or together with quercetin (10 μM) for 24 hours. Data was normalised to untreated control (FPN1A alone without any treatment). Data analysed by one-way ANOVA and Dunnett's post-hoc test. N=6; Data presented as mean ± SEM.

5.5. Discussion

DMT1, Nramp1, TRPML1 and TRPML2 are divalent metal transporters expressed in the phagolysosomal membranes of macrophages and are suggested to be involved in recycling iron from senescent erythrocytes and trafficking iron through the macrophage (Gunshin et al. 1997, Kuhn et al. 1999, Dong et al. 2008, Soe-Lin et al. 2008). Ferroportin (FPN) is the only known cellular iron exporter. These results demonstrated that most, but not all of these THP1 iron transporters are at least partly regulated by intracellular iron concentrations.

This section of the study was designed to investigate whether changes in cellular iron concentration altered the expression of THP1 macrophage iron transporters. It was predicted that increasing cellular iron with the addition of hemin (50μM) would decrease expression of iron transporters so as to stop iron overload. Conversely, a decrease of iron by the iron chelator quercetin would increase the gene expression of these transporters. As predicted, hemin caused a significant reduction in the mRNA expression of Nramp1, TRPML1, TRPML2 and FPN (Figure 5.2).

Nramp1 (Natural resistance-associated macrophage protein 1) is expressed in the membrane fraction of macrophages (Vidal et al. 1996) with highest expression being in circulating macrophages and monocytes (Cellier et al. 1994). As a divalent transporter, it is thought to transport Mn^{2+} and Co^{2+} as well as Fe^{2+} (Kuhn et al. 1999) however there is controversy as to the direction of iron transport with no general consensus being reached. On the one hand, Nramp1 has been reported to transport

iron into phago-lysosomes (Kuhn et al. 1999) but on the other, studies suggest Nramp1 is involved in iron efflux out of phago-lysosomes and even out of cells (Biggs et al. 2001, Mulero et al. 2002). Our results show an inverse relationship of decreased Nramp1 expression with increased iron concentration which is more consistent with a role of Nramp1 as an efflux protein in the phago-lysosmal membrane involved in macrophage iron trafficking. The inverse correlation found contradicts others who have found a parallel relationship between Nramp1 protein expression and iron levels in macrophages (Atkinson and Barton 1999, Baker et al. 2000, Soe-Lin et al. 2008) but these studies often use a different strain of macrophage such as murine RAW264.7 or J774 which may account for the differences. These cell lines originate from Nramp1^{D169} mice and do not produce mature Nramp1 protein (Vidal et al. 1996). Transfection with exogenous Nramp1 as in Soe-Lin et al (2008) (RAW264.7 transfected with Nramp1^{G169}) is unlikely to produce the same effects as endogenous Nramp1 in Thp1 macrophages.

Dong et al. (2008) recently demonstrated that transient receptor potential mucolipin 1 (TRPML1) is ubiquitously expressed and encodes for an iron release channel found in the late endosome and lysosome. Lysosomes are components of the endocytic pathway that digest the cellular material delivered to autophagosomes during cell renewal and death (reviewed in (Luzio et al. 2005)). The role of the TRPML channels has not yet been looked at in human macrophages.

We found that both TRPML1 and TRPML2 were expressed in macrophages and their expression was regulated in a similar manner by being significantly reduced with

hemin treatment suggesting a similar type of role to Nramp1 of iron efflux. TRPML1-deficient fibroblast cells exhibit lysosomal iron overload as well as cytosolic iron deficiency which is consistent with a role of TRPML1 in endolysosomal iron release (Dong et al. 2008). However with evidence that TRPML1 is also permeable to Ca^{2+} (LaPlante et al. 2004) this makes it unlikely that TRPML1 is solely dedicated to iron metabolism.

DMT1 was the only transporter that was not altered by hemin treatment. One possible reason for this is that hemin presents iron in a form that is inaccessible to DMT1. The use of hemin for cellular iron loading is based on the assumption that following erythrophagocytosis, or hemin treatment, haem is catabolised within the phagolysosome by haem oxygenase. It has recently been shown that DMT1 may not be recruited to the erythrophagosomal membrane (Delaby et al. 2012) suggesting that DMT1 may be involved in iron acquisition in macrophages via the transferrin-transferrin receptor cycle rather than recycling of haem iron from haemoglobin. In addition the primers used in this study to amplify DMT1 are common to all DMT1 isoforms. The use of isoform-specific primer sets would allow us to explore more fully the effect of iron on DMT1 expression.

Our results showed that in response to quercetin (1 – 100 μM), DMT1 and TRPML1/2 expression only altered significantly with 100 uM quercetin (Figure 5.1). However, at this concentration quercetin could be affecting cell viability (as observed in HepG2 cells). In future, a Trypan blue exclusion test or a cell viability assay should be

performed to see if this is the underlying reason for the dramatic decrease in expression.

In contrast to the other transporters investigated, quercetin caused a significant dose-dependent increase in FPN expression. This was surprising as quercetin decreased FPN expression in both Caco-2 and HepG2 cell lines. We therefore transfected Thp1 macrophages with 5'FPN promoter constructs to examine whether quercetin was having any effect on promoter activity in Thp1 cells. We found that quercetin (10 μM) did indeed significantly induce both FPN1A and 1B promoter activities, whereas hemin had no effect. In contrast, in Caco-2 cell line quercetin had no effect on FPN1A or 1B. This data strongly implies that quercetin stimulates the FPN promoter to increase FPN expression independent of iron levels and that this effect is specific only to macrophages. This demonstrates the cell-specific effects of quercetin and that FPN is differentially regulated dependent on cell/tissue. Differential effects on FPN expression in enterocytes and macrophages have been previously reported in response to hepcidin (Chaston et al. 2008, Chung et al. 2009). These studies showed that hepcidin rapidly decreased FPN expression in mouse splenic macrophages and Thp1 cells but had no effect on mouse duodenal enterocyte or Caco-2 cell FPN.

Quercetin may upregulate FPN in order to maintain the demands of erythropoiesis. However when we looked at Epo stimulation of FPN, the stimulatory effect on FPN promoter observed in Caco-2 cells was not seen in Thp1. Similarly, inhibiting the hypoxia inducible factors, HIF1α and HIF2α, which are also activated by iron deficiency and erythroid requirement for iron, had no effect on FPN promoter activity.

It is possible that quercetin acts by stimulating Nrf2 activity in macrophages. Nrf2, through binding to antioxidant responsive elements in the 5'promoter has been shown to increase FPN expression in macrophages (Marro et al 2010). Future studies should explore this pathway in more detail.

Our work in the Thp1 cell linepresents exciting new possibilities that quercetin regulates macrophage iron metabolism. Macrophages are the major contributor to body iron turnover, recycling approximately 25 mg iron/day from red blood cells. Factors which can regulate this turnover might be important therapeutically for treating a number of iron-related disorders. We have shown that TRPML1 and TRPML2 expressed in human THP1 macrophages have an inverse relationship with cellular iron concentration. These iron transporters as well as Nramp1 and FPN, but not DMT1, are regulated by iron. FPN shows an additional regulation by quercetin which uses the 5'UTR promoter to increase FPN expression. Whether the stimulatory effect of quercetin is able to override the inhibitory iron signal is yet to be determined.

6. Conclusion

6.1. General conclusion

Systemic iron homeostasis requires a meticulous coordination of intestinal iron absorption, efficient recycling of iron from senescent erythrocytes and controlled storage of iron in hepatocytes and macrophages (Andrews 2008). This thesis investigated the regulatory effects of one of the most dominant dietary polyphenols, quercetin, on all three aspects of iron metabolism and investigated its mechanism of action.

In Caco-2 cells we showed that quercetin significantly down-regulated FPN gene and protein expression. Through miRNA array screening and validation studies, we have identified the miRNA hsa-miR-17-3p as a novel candidate for quercetin-induced FPN regulation. Previous work in the field has shown that miRNAs can regulate gene expression of iron transporters in other cell types such as miR-Let-7d for DMT1(-IRE) in erythroid cells (Andolfo et al. 2010) and miR-485-3p for FPN in hepatocyte and erythroid cells (Sangokoya et al. 2013). Computational analyses suggest that miRNAs may regulate up to 30% of human protein-coding genes (Lewis et al. 2005) although only a very small number of target genes have actually been experimentally confirmed.

Mechanistically, miRNAs repress translation and/or mediate RNA degradation (Wu et al. 2006). Therefore activation of miR-17-3p by quercetin may regulate FPN by causing mRNA degradation and/or preventing RNA translation explaining low levels of both FPN gene and protein with increased quercetin treatment. miRNAs tend to be

cell-specific (Castoldi et al. 2011, Andolfo et al. 2010) and interestingly quercetin increased FPN expression in macrophages rather than decrease as it did in Caco-2 and HepG2, suggesting that quercetin is using different regulatory mechanisms. This highlights that FPN is differentially regulated dependent on tissue type and is supported by *in vivo* work showing that FPN in macrophages and enterocytes responds differently to hepcidin challenge (Chaston et al. 2008, Knutson et al. 2005, Chung et al. 2009).

Quercetin treatment on HepG2 cells showed a stimulation of hepcidin mRNA expression. As the site of hepcidin synthesis, this increased production could provide an additional means of inhibiting intestinal iron absorption via FPN degradation. Quercetin also exhibited both pro- and anti- proliferative/apoptotic effects; this was conditional on the type of iron used (Leimberg et al. 2008, Alcantara et al. 1991) and concentration of quercetin used (Shih et al. 2004, Granado-Serrano et al. 2006).

Taken together, these results show various mechanisms at play and emphasise the potential benefits of quercetin on iron-related disorders (Figure 6.1). In iron overload situations, inhibition of intestinal FPN by quercetin would limit dietary absorption of iron. Increased hepatic hepcidin production would further help to limit iron uptake (Nemeth et al. 2004b). However as quercetin is shown to stimulate Thp1 FPN, quercetin may counteract the effects of increased hepcidin specifically in macrophages to ensure that a constant and consistent iron recycling system is in place to maintain iron supply for erythropoiesis. This would be particularly useful in conditions associated with anaemia of chronic disease where macrophages have high iron

retention but a lack of iron export results in reduced erythropoiesis (Weinstein et al. 2002). Quercetin may also have positive patho-physiological effects. In iron overload diseases such as hereditary haemochromatosis, iron accumulation often occurs in the liver (Nancy C. Andrews 1999). Our results show that FAC decreased hepatocyte cell viability, which *in vivo* could lead to fibrosis; whereas exposure to haem increased proliferation which could be pro-tumourogenic. Addition of quercetin was able to counteract both of these effects highlighting the potential beneficial effects of quercetin on liver physiology.

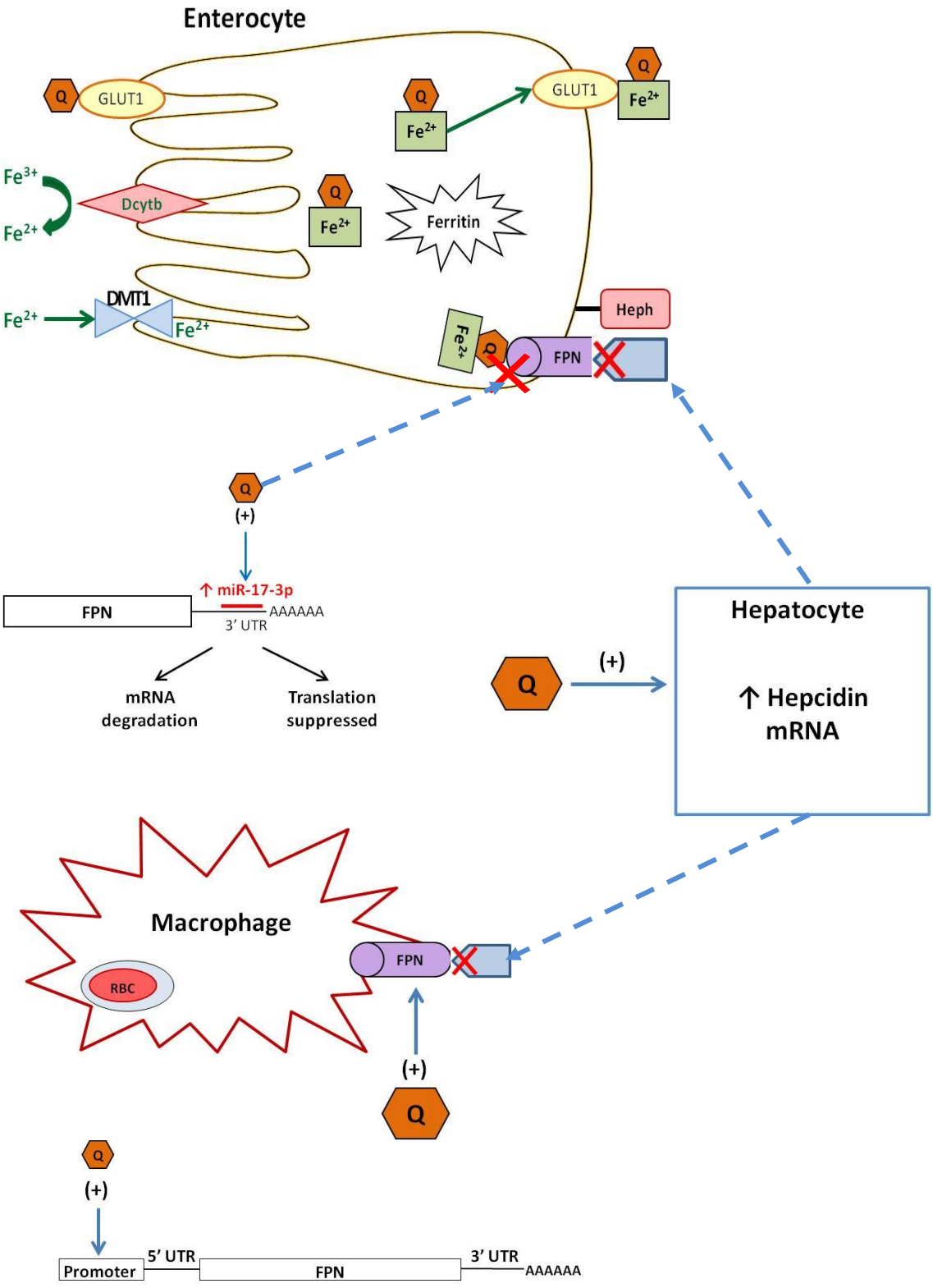


Figure 6.1 Proposed working model for the regulatory actions of quercetin on iron metabolism

6.2. Further study

This work has investigated, discovered and proposed mechanisms of quercetin action on iron metabolism in well established cell lines. Similar studies need to be carried out *in vivo* in animal models to confirm these effects and should be followed by human intervention studies to determine any potential benefits for groups at risk of developing iron metabolism-related disorders.

Having looked at 5'FPN promoters as a means of FPN regulation, we did not find any quercetin interaction. However, the promoter tested was only a small fragment (895/1050 bp) and it could be that quercetin interacts with FPN further upstream therefore further work exploring the whole 5'UTR sequence is warranted for possible sites of quercetin regulation.

We showed that recombinant erythropoietin (Epo) stimulation of FPN gene expression could be blocked by quercetin in Caco-2 cells, suggesting that quercetin was affecting FPN expression by an Epo-stimulatory pathway. Focusing on the PI3K pathway we found that quercetin and LY294002 (a PI3K inhibitor) had similar effects in support of this hypothesis. However phospho-Akt, a downstream target of PI3K, is also a protein of interest as its phosphorylation is increased by Epo. Western blotting should be performed to see whether both quercetin and LY294002 inhibit phosphorylation of Akt to confirm they are working by the same pathway. Furthermore, in addition to PI3K/Akt pathway, phosphorylated Epo-R activates multiple downstream kinase pathways including JAK2 and MAPK (Marzo et al. 2008) and it might be that

quercetin is affecting these (Kong et al. 2000). Specific kinase inhibitors should be used in cell models to examine their effects on FPN and then compared to Epo and quercetin responses as indication of shared pathways for FPN regulation.

This study noted that hsa-miR-17-3p was upregulated by quercetin and may be responsible for FPN downregulation in intestinal cells, thus a hsa-miR-17-3p mimetic should be used to block this pathway in the Caco-2 model to see if FPN mRNA down-regulation is also inhibited. In mouse models, this miRNA, along with other miRNAs possibly involved in regulation of iron metabolism genes, should be validated by q-PCR and similar experiments with mimetics/inhibitors should be performed.

Several mouse models of iron-overload have been developed over the years including Hfe knockouts (Hfe −/−) (Fleming et al. 2001), β-thalassaemic mice (Raja et al. 1994) and hepcidin knockout mice (Hepc1–/–) (Nicolas et al. 2001, Lesbordes-Brion et al. 2006). Strategic use of these mouse models in dietary feeding studies giving quercetin at a range of nutritionally acceptable doses (0.006 - 0.06% (w/w) corresponding to an equivalent human intake of 30 - 300 mg/day) (Milenkovic et al. 2012) would allow an assessment of how dietary polyphenols might interact with iron sensing, storage, transport and recycling. These studies may identify novel pathways that could be exploited for future development of therapies to treat disorders of iron homeostasis.

The ultimate goal is to explore the therapeutic effects of quercetin and trial it as an iron chelator and modifier of iron absorption in human studies, focusing on patients with iron-overload diseases. As a natural occurring compound in the diet, effective doses of quercetin should be well-tolerated and could provide an adjunct to current

therapies. The effect of quercetin on miRNAs also opens up the possibility of miRNA inhibitors as tools for iron therapy in selected target tissues/organs.

7. References

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